

2012

Purification and Characterization of Blood Aspirin Hydrolases

Gang Zhou
Cleveland State University

Follow this and additional works at: <https://engagedscholarship.csuohio.edu/etdarchive>

 Part of the [Chemistry Commons](#)

How does access to this work benefit you? Let us know!

Recommended Citation

Zhou, Gang, "Purification and Characterization of Blood Aspirin Hydrolases" (2012). *ETD Archive*. 325.
<https://engagedscholarship.csuohio.edu/etdarchive/325>

This Dissertation is brought to you for free and open access by EngagedScholarship@CSU. It has been accepted for inclusion in ETD Archive by an authorized administrator of EngagedScholarship@CSU. For more information, please contact library.es@csuohio.edu.

PURIFICATION AND CHARACTERIZATION OF BLOOD ASPIRIN
HYDROLASES

by

Gang Zhou

A dissertation submitted to faculty of

The Cleveland State University

in partial fulfillment of requirements for the degree of

DOCTOR OF PHILOSOPHY

IN

CLINICAL/BIOANALYTICAL CHEMISTRY

Department of Chemistry

Cleveland State University

April 2012

© Copyright by Gang Zhou 2012

All Rights Reserved

This dissertation has been approved for
the Department of Chemistry and for
the College of Graduate Studies of
Cleveland State University

By

Date _____
Dr. Thomas McIntyre, Dept. of Cell Biology/Cleveland Clinic, LRI
Major Advisor, Advisory Committee Chair

Date _____
Dr. Martha Cathcart, Dept. of Cell Biology/Cleveland Clinic, LRI
Advisory Committee Member

Date _____
Dr. Donald Jacobsen, Dept. of Cell Biology/Cleveland Clinic, LRI
Advisory Committee Member

Date _____
Dr. Jun Qin, Dept. of Molecular Cardiology/Cleveland Clinic, LRI
Advisory Committee Member

Date _____
Dr. Xue-Long Sun, Dept. of Chemistry/Cleveland State University
Advisory Committee Member

Date _____
Dr. Crystal Weyman, Dept. of BGES/Cleveland State University
Advisory Committee Member

ACKNOWLEDGMENTS

First I would like to thank Dr. Thomas McIntyre for giving me the opportunity to work in his group and working on such an interesting thesis project. Dr. Thomas McIntyre is a great scientist. His feedback and input were vital for this shy foreign graduate student to gain appreciation and confidence in the scientific process, and my ability to use it to ask and answer relevant and important questions and to survive my Ph.D.. Also, Dr. Thomas McIntyre's quick wit and sense of humor made working in the lab very enjoyable. Without the guidance, patience, and encouragement from him, I cannot complete this project in four years.

I wish to acknowledge the contribution of many people to the development of this thesis research project and my scientific abilities. I would like to thank all members of McIntyre Lab. I especially thank Dr. Gopal Marathe for training me techniques and early involvement in this project, and lots of help on research. I thank Dr. Stanley Hazen for providing serum samples and Dr. Hooman Allayee for genome-wide data analysis. Also I thank Dr. Oksana Lockridge for providing BChE null plasma. I thank Mark Calabro and Erin Brady for plasma and erythrocytes isolation and materials ordering. I thank Dr. Thomas Tallant for providing FPLC support. I also thank Dr. Belinda Willard for mass spectrometry.

I also want to thank all committee members for valuable advice and encourage. This project cannot be done without their efforts. I thank Dr. Martha Cathcart for her scientific and circumspect guidance, which made me learn more

about science; thank Dr. Donald Jacobsen for his suggestions and encouragement; thank Dr. Jun Qin for his kind advice; thank Dr. Xue-long Sun for his valuable guidance and encouragement; thank Dr. Crystal Weyman for her encouragement, instruction, and enlightening in my Ph.D. study.

Finally, I wish to thank my family for their love and support. I thank my parents for everything in my life and appreciate my wife's company and support and selfless contribution to this project. Without their understanding and support this project cannot be done.

PURIFICATION AND CHARACTERIZATION OF BLOOD ASPIRIN HYDROLASE

GANG ZHOU

ABSTRACT

Aspirin prophylaxis suppresses major adverse cardiovascular events, but its turnover in blood is rapid, which limits its inhibitory effects on platelet cyclooxygenase and thrombosis. Inter-individual variability of platelet responses to aspirin clinically presents as treatment failure, and this increasing clinical phenomenon is frequently named aspirin “resistance”. However, the molecular mechanisms behind this are unclear. Blood aspirin hydrolases are believed to control aspirin survival in vivo, but the identity of the circulating enzyme(s) that hydrolyzes aspirin remains unknown.

In this thesis, blood aspirin hydrolases were identified and characterized. The relationship between blood aspirin hydrolases and aspirin efficacy was investigated. RP-HPLC analysis of the product salicylic acid with an internal standard showed plasma hydrolysis of aspirin varied 12-fold variation among 2,275 individuals. Genome-wide association analysis using serum aspirin hydrolytic activity from 2,275 individuals showed a genetic component to aspirin hydrolytic variation, and that only BChE significantly associated to aspirin variation. However, plasma from an

individual with an inactivating point mutation in BChE effectively hydrolyzed aspirin. A non-BChE aspirin hydrolase was found in plasma, which can be distinguished from BChE by procainamide and oxidized ATP. Erythrocyte aspirin hydrolase was purified by 1400-fold and type I PAF acetylhydrolase was identified as a candidate aspirinase by mass spectrometry. Recombinant PAFAH1B2 hydrolyzed aspirin and aspirin was effectively hydrolyzed in cells ectopically expressing PAFAH1B2 and PAFAH1B3. Type I PAFAH also accounts for non-BChE plasma aspirin hydrolytic activity. Both plasma and erythrocytes were found modulated aspirin efficacy significantly, which varied by aspirin hydrolytic activity variation.

I hope my work in this thesis will help to understand aspirin “resistance” and shed light on personalized medicine of aspirin.

Table of Contents

ABSTRACT	vi
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xii
CHAPTER I.....	1
1. INTRODUCTION	1
1.1. A history of aspirin	1
1.2. Properties and functions of aspirin.....	4
1.3. Metabolism of aspirin	6
1.4. Platelets	9
1.5. Aspirin resistance	16
1.6. Aspirin esterase	21
CHAPTER II.....	24
2. MATERIALS and METHODS	24
CHAPTER III	40
3. CHARACTERIZATION AND IDENTIFICATION OF HUMAN PLASMA ASPIRIN HYDROLASES	40
3.1. Introduction	40
3.2. Results.....	45
3.3. Discussion	72
CHAPTER IV	78
4. IDENTIFICATION AND CHARACTORIZATION OF ASPIRIN HYDROLASE IN HUMAN ERYTHROCYTES	78
4.1. Introduction	78
4.2. Results.....	81
4.3. Discussion	104
CHAPTER V	110
5. ELUCIDATION OF THE RELATIONSHIP BETWEEN ASPIRIN HYDROLASES AND ASPIRIN EFFICACY	110
5.1. Introduction	110

5.2. Results.....	112
5.3. Discussion	122
CHAPTER VI	125
6. CONCLUSIONS AND DISCUSSIONS	125
7. BIBLIOGRAPHY	132

LIST OF FIGURES

Figure 1	3
Figure 2	3
Figure 3	5
Figure 4	8
Figure 5	16
Figure 6	19
Figure 7	20
Figure 8	26
Figure 9	27
Figure 10	29
Figure 11	30
Figure 12	31
Figure 13	32
Figure 14	33
Figure 15	46
Figure 16	47
Figure 17	48
Figure 18	49
Figure 19	51
Figure 20	52
Figure 21	54
Figure 22	56
Figure 23	57
Figure 24	58
Figure 25	59
Figure 26	62
Figure 27	64
Figure 28	65
Figure 29	66
Figure 30	68
Figure 31	69
Figure 32	70
Figure 33	71
Figure 34	82

Figure 35	83
Figure 36	84
Figure 37	86
Figure 38	89
Figure 39	91
Figure 40	92
Figure 41	93
Figure 42	94
Figure 43	95
Figure 44	96
Figure 45	97
Figure 46	98
Figure 47	100
Figure 48	101
Figure 49	103
Figure 50	113
Figure 51	115
Figure 52	116
Figure 53	118
Figure 54	119
Figure 55	121

LIST OF ABBREVIATIONS

ACD	Acid Citrate Dextrose
ADP	Adenosine diphosphate
APEH	Acylpeptide hydrolase
ASA	Acetylsalicylic Acid
ATP	Adenosine triphosphate
BChE	Butyrylcholinesterase
COX	Cyclooxygenase
CVD	Cardiovascular Disease
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HBSS	Hanks Buffered Saline Solution
HDL	High Density Lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid chromatography–mass spectrometry
LDL	Low Density Lipoprotein
MAFP	Methyl arachidonyl fluorophosphonate
NSAID	Non-Steroidal Anti-Inflammatory Drugs
ODS	Octadecylsilane
PA	Phenol acetate
PAF	Platelet-Activating Factor

PAFAH	Platelet-Activating Factor Acetylhydrolase
PBS	Phosphate Buffered Saline
PGG	Prostaglandin G
PON	Paraoxonase
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PVDF	Polyvinylidene Fluoride
SA	Salicylic Acid
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SNP	Single Nucleotide Polymorphism
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline Tween 20
TxA	Thromboxane
UV	Ultraviolet

CHAPTER I

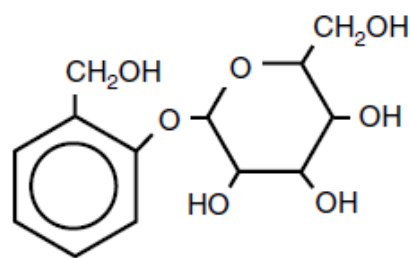
1. INTRODUCTION

1.1. A history of aspirin

Human beings are known to have more than 100,000 years of history, but knowing therapies using naturally occurring plants and derivatives of plants is just about thousands years old. Aspirin is the oldest and most widely used medicine in the world. Almost everyone knows or has the experience of taking aspirin, but not everyone knows its history. People in early days, especially the Assyrians and the ancient Egyptians almost 2,000 years ago, recorded using willow bark and leaves to alleviate arthritis pain (Jack 1997). However, at that time they were unaware of the reasons behind this. About 100 years ago, Hippocrates, the Greek physician, described and documented that willow bark has analgesic function. The anti-inflammatory and anti-pyretic effects of willow bark were first documented by Galen. However, Edward Stone is generally regarded as the first person who gave the scientific description of willow bark in clinical use, and he also pointed out the anti-malarial effects of willow bark (Jack 1997; Hawkey 2005).

With rapid development of chemistry in the 18th and 19th centuries, Henri Leroux, a French Pharmacologist, and Raffaele Piria, an Italian Chemist, isolated a

functional compound from willow bark in 1826. Almost at the same time, the same compound was purified by Johann Buchner (Jack 1997), and was named salicin (Figure 1), which means “willow” in Latin. Purification and isolation of salicin from willow barks was optimized at a later time. In the meantime, physicians started prescribing salicin as a drug to relieve fever and pain. In 1876, Thomas Maclagan, a Dundee physician, published a paper to report the beneficial effects of salicin in treating patients who had rheumatism in the well reputed British Journal “The Lancet” (Maclagan 1876). Ten years later, Herman Kolbe, a German Chemist, first synthesized salicylic acid from salicin. German chemist, Felix Hoffmann, first synthesized according to available records and commercialized the acetylsalicylic acid in 1897 (Jack 1997; Miner and Hoffhines 2007; Schrör 2009). Because his farther hated taking salicylic acid, which irritated his gut, he wanted to make a substitute for salicylic acid. And finally he was able to create acetylsalicylic acid from salicylic acid (Figure 2), which was named aspirin. At that time, he worked at Bayer, and with a pharmacist, Heinrich Dreser, together they did the pharmaceutical and clinical trial for aspirin. On 23 January 1899 they decided to give this compound the brand name “Aspirin” and finally, on March 6th 1899, it was registered at the Kaiserlichen Patentsamt, Berlin and received a patent. And then Bayer put aspirin on the market as an analgesic drug for rheumatism (Hawkey 2005). Aspirin is the first synthesized drug and its development is also regarded as the milestone of the modern pharmaceutical industry.



Salicin
(2-[Hydroxymethyl]phenyl- β -D-glycoside)

Figure 1. Structure of Salicin

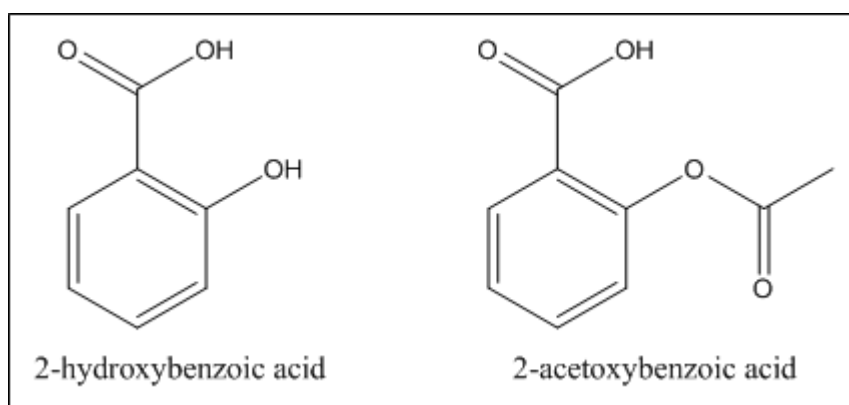


Figure 2. Structures of Salicylic acid and Aspirin. Aspirin has an acetyl group, which make aspirin different from salicylic acid.

Due to better tolerance to the stomach over salicylic acid, aspirin quickly became popular and was widely used all over the world as a substitute for salicylic acid. Until now aspirin still is a famous medicine, although it has side effects, such as gut bleeding. Structurally, acetyl group is the only difference of aspirin from salicylic acid. And because of this, aspirin has a novel ability to prevent cardiovascular events (Weiss 2003), which is first discovered by Dr. Lawrence L. Craven. However,

patients taking aspirin as a prophylactic agent were still subject to heart attacks (Helgason, Bolin et al. 1994; Bradbury 2002).

Despite its routine use, the mechanisms of aspirin action were poorly understood. At meantime, Sir John Vane and Priscilla Piper were studying the inhibitory affects of aspirin on the biosynthesis of prostaglandins, which eventually explained how aspirin works. In 1982, John Vane won the Nobel Prize in Physiology or Medicine because of his great work on aspirin. However, the aspirin story is not over. It was found that aspirin possibly has therapeutic value to a variety of diseases, including dementia and certain cancers. But the mechanisms behind these new findings are not still clear and needed further investigation, although aspirin is used as a prophylactic agent.

1.2. Properties and functions of aspirin

Chemically synthesized aspirin is a white powder with better solubility in ethanol (20%) than in water (0.3%). The solubility of aspirin in aqueous media largely depends on pH value (Figure 3). When the pH is around 7, there is a minor variation in aspirin hydrolysis. This property makes measurement of aspirin hydrolysis reproducible at physiological pH.

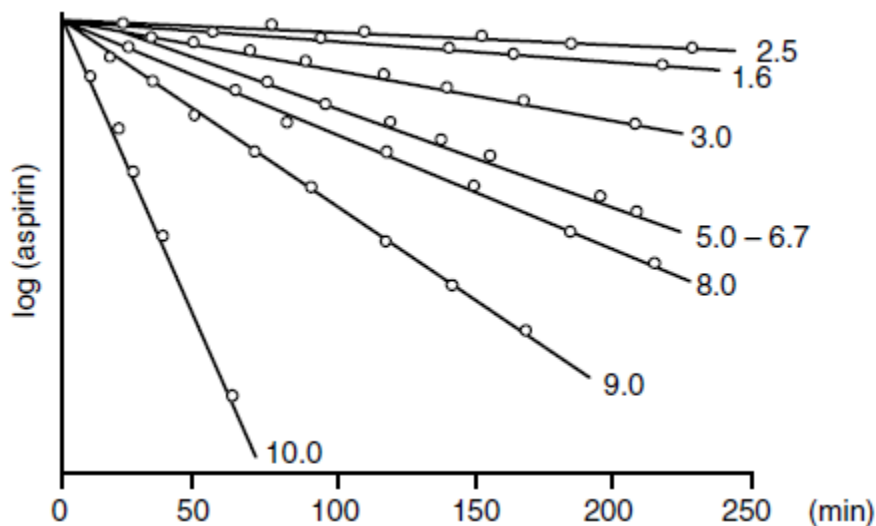


Figure 3. Aspirin (1.5mM) hydrolysis in aqueous solution at different pH. (Horsch 1979). Numbers in the figure are pH values. (42°C)

Aspirin has multiple beneficial effects, such as analgesic, antipyretic, and anti-inflammatory functions. The reason for aspirin's ability to display multiple effects is not clear. Once aspirin gets into blood, it is hydrolyzed and becomes a mixture of several components, intact aspirin, salicylic acids, their metabolites, and acetic acid, which give aspirin pharmacological activities via both intact molecules and salicylic acids. This may affect certain cellular targets and signaling pathways. The main functions of aspirin as mentioned above focused on three aspects: 1) the original clinical function as a classic Non-Steroidal Anti-Inflammatory Drug (NSAID) is analgesic and antipyretic activity; 2) the anti-inflammatory effect is also a valuable clinical function. 3) the effect of aspirin in anti-platelet function. Sir John Vane in 1971 first gave the pharmacological explanation for aspirin's multiple actions as blocking prostaglandin biosynthesis (Vane 1971). Because of these important findings, he was awarded Nobel Prize in Physiology and Medicine in 1982. His findings gave solid evidence that blocking prostaglandins production is the basis reason of the

analgesic, anti-inflammatory and anti-platelet effects. Acetylation of salicylic acid added a new property to aspirin (Figure 2) not showing in salicylates, inhibition of platelet function. Cardiovascular disease prevention effects of aspirin stems from its anti-platelet function. Aspirin's anti-platelet effects are believed to be due to acetylation of cyclooxygenase-1 (COX-1) at 530 serine residue (Roth and Majerus 1975). The acetylation irreversibly inhibits COX-1 in anucleated platelets, thereby blocking thromboxane A₂ production and suppressing platelet activation. Salicylic acid, the metabolite of aspirin hydrolysis, is reported to inhibit COX-1 as well, but the potency of salicylic acid in inhibiting COX-1 is very low when compared to that of aspirin and it also varies with albumin concentrations (Warner, Vojnovic et al. 2006).

In addition to these major functions, there are other reported effects of aspirin. Researchers are still making efforts to find novel mechanisms for aspirin through further understanding of its molecular action.

1.3. Metabolism of aspirin

The metabolism of aspirin *in vivo* involves two distinct processes. The two processes are thought to occur independently to each other. Pharmacologically the two processes are called phase I and II biotransformation. Phase I refers to aspirin losing its acetyl group. In this phase, aspirin is undergoing hydrolysis and nonspecific transferring of the acetyl group and produces salicylic acid. Phase II biotransformation refers to the further transforms of salicylic acid. In this phase, salicylic acid is conjugated to other soluble molecules, which can be eliminated by kidney. Different biological significant enzymes may involve in the two independent processes.

Aspirin after oral administration is metabolized in stomach, liver, and blood and broken down to salicylic acid and acetic acid. Blood is believed to be the major place where aspirin gets hydrolyzed (70%) after oral administration. The half-life of aspirin *in vivo* is only 15-20 minutes (Pedersen and FitzGerald 1984), while the half-life of salicylate, aspirin hydrolysis product, under therapeutic doses is 2-4h, but can vary with increased doses (Done 1960). Approximately 80% of salicylates are conjugated with glucuronic acid forming phenolic glucuronide and salicyl CoA and with glycine forming salicyluric acid in the liver (Figure 4). Salicylates can be hydroxylated to gentisic acid as well. However, only a limited amount of salicylic acid is metabolized by these pathways. Given large doses of salicylic acid, the kinetics of metabolism will switch from first order to zero order (Levy and Tsuchiya 1972).

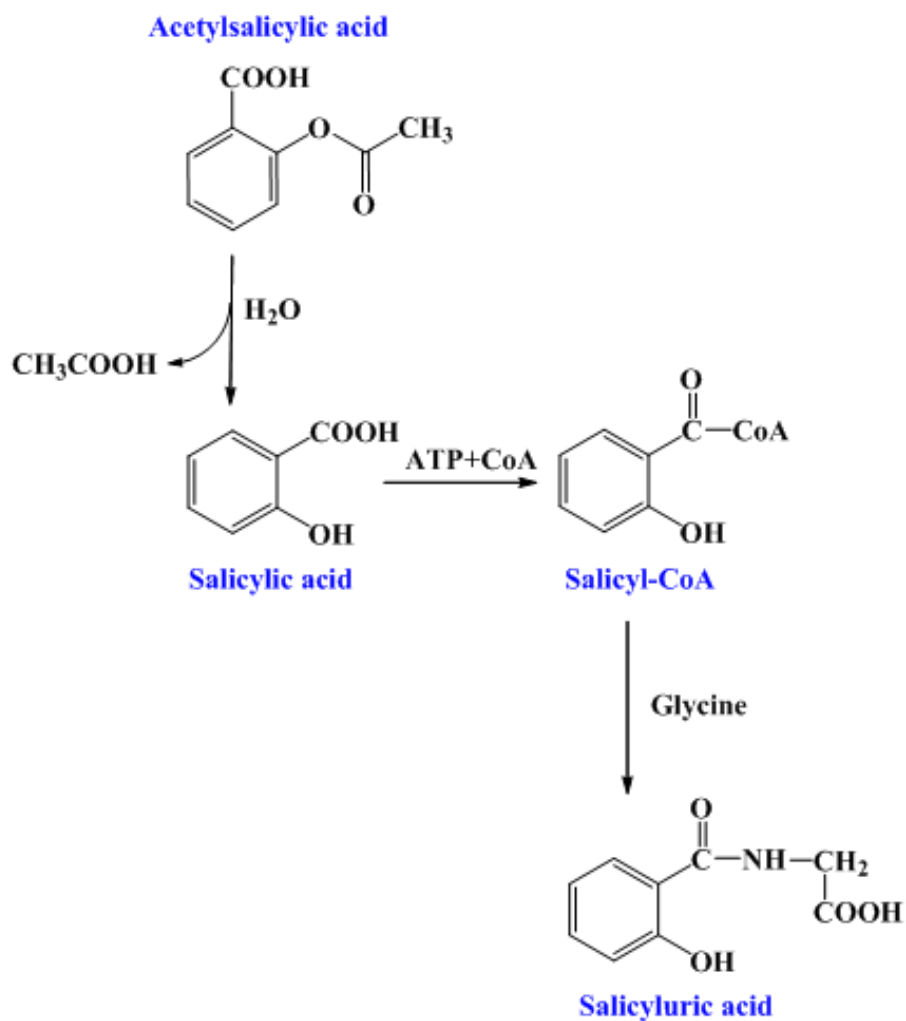


Figure 4. Catabolism of aspirin *in vivo*. Aspirin is catabolized to salicylic acid and then to salicyluric acid.

Salicylic acid is mainly excreted by kidney; 75% as salicyluric acid; 10% as free salicylic acid; 10% as salicylic phenol; 5% as acyl glucuronides; and gentisic acid is less than 1%. When an adult ingests less than 250 mg salicylic acid, half-life of salicylic acid will be 2-3 hours and all metabolic processes are in first order kinetics (Hartwig-Otto 1983). When more than 4g salicylic acid is ingested, because the salicyl phenolic glucuronide and salicyluric acid biotransformative pathways become saturated, the half-life of salicylates increases to 15-30 hours (Levy 1965). Because

when pH value is above 6 in urine, excretion of salicylic acid by kidney becomes more important when the biotransformative pathways become saturated (Hollister and Levy 1965). So urinary alkalization is usually a measure used to increase elimination of over loaded salicylates.

1.4. Platelets

Platelets, or thrombocytes, are small and anucleate cells. They have a diameter of 2–3 μm . Platelets are derived from megakaryocytes. Platelets have a short lifespan in circulation, which is normally 7 to 10 days in average before being removed by the hemopoietic tissue spleen. Platelets have a single peripheral microtubule coil (Fitzgerald, Poncz et al. 1987) and a subplasmalemmal membrane skeleton of actin (Zimrin, Eisman et al. 1988). Platelets circulate in blood, and participate in hemostasis and blood clotting. They also produce growth factors.

1.4.1. Platelet activation

Resting platelets do not interact with other cells. However, they are ready to respond and react if they are subjected to certain stimuli. The reaction of platelets is protective, but sometime it is destructive when losing control. When underlying connective tissue elements such as collagen are exposed during vascular damage, platelets around the lesion will be activated, which will induce a sequence of events including adhesion to collagen matrix, a change of platelet morphology from a disk to

an irregular sphere with filopodia, granule secretion, and aggregate formation. These events are usually referred to as adhesion, shape change, secretion, and aggregation.

Because of their roles in physiology, platelets are easy to be activated. They can be stimulated by several agonists, such as collagen, von Willebrand factor (vWF), fibrinogen, thrombin, Adenosine diphosphate (ADP), epinephrine, vasopressin, platelet activating factor (PAF), and thromboxane A_2 (TxA_2). These agonists are commonly classified as strong and weak. In order to respond quickly to vascular and tissue damage, platelets can amplify stimulating signals to self-reinforce reactions. This is a positive feedback loop, which once started cannot be stopped and also more and more strong. Two intracellular pathways are involved in platelet activation. One is phosphoinositide pathway, the other is eicosanoid pathway (Ronald Hoffman; Edward J. Benz 1991). In the phosphoinositide pathway after stimulation phospholipase C gamma (PLC) cleaves phosphatidylinositol 4, 5-bisphosphate (PIP_2) in platelets or other cells to two second messengers, inositol 1, 4, 5-trisphosphate (1, 4, 5- IP_3) and diacylglycerol (DAG). These second messengers increase intracellular calcium concentration which then stimulates other signaling events including Protein Kinase C. In eicosanoid pathway, membrane phospholipid hydrolysis is catalyzed by phospholipase A_2 to liberate arachidonate, which is then further metabolized to PGH_2 by cyclooxygenase-1 or 2 and is then catalyzed by thromboxane synthase in platelets to thromboxane A_2 (TxA_2), which reacts with its specific receptors to enhance the reaction. Because TxA_2 can easily diffuse across the plasma membrane of platelet, it can serve as a lipid mediator among platelets as well as within platelets. The

metabolism pathway of arachidonate converting to TxA_2 is selectively inhibited by nonsteroidal anti-inflammatory drugs (NSAID), especially aspirin.

The final consequence of platelet activation is platelet aggregation. There include a glycoprotein IIb-IIIa complex that belongs to a family of adhesive protein receptors called integrins on the plasma membrane, which serve as platelet fibrinogen receptors. Platelets form aggregates which need fibrinogen to bridge adjacent platelets. *Ex vivo* platelet aggregation is usually used to examine platelet function by monitoring light transmission for washed platelets or platelet rich plasma (PRP), or the electrode impedance of whole blood.

1.4.2. Platelet- activating factor (PAF)

Platelet activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is one of the molecules produced by cells in and around the vascular space which stimulates platelet activation. It was first described as a platelet activator released from stimulated mast cells, although its biological effects are beyond platelet activation (Braquet, Bourgain et al. 1989; Page and Abbott 1989). PAF is produced by neutrophils, macrophages, eosinophils, and endothelial cells, but minimally by human platelets (Braquet, Touqui et al. 1987; Chao and Olson 1993; Ishii and Shimizu 2000). PAF is the first bioactive phospholipid characterized to have an effect on platelets (Benveniste, Tence et al. 1979; Blank, Snyder et al. 1979; Demopoulos, Pinckard et al. 1979), so it has attracted a lot of attention.

As a potent mediator, PAF can elicit significant biological responses at nanomolar concentrations both *in vivo* and *ex vivo* (Snyder 1995). PAF activates platelets and causes aggregation and secretion at concentrations ranging from 10 to 100 nM (Lapetina and Siegel 1983; MacIntyre and Pollock 1983; Klopogge and Akkerman 1984). Binding studies with radiolabeled PAF are complicated by the ability of many cells, including platelets, to take up and metabolize the mediator (Klopogge and Akkerman 1984; Braquet, Touqui et al. 1987; Valone 1988). But the dissociation constants that have been reported are at least as low as the concentrations required to activate platelets (Klopogge and Akkerman 1984; Ukena, Dent et al. 1988; Valone 1988). PAF has been shown to cause shape change, phosphoinositide hydrolysis and protein phosphorylation in platelets and to increase the cytosolic free calcium concentration (Lapetina and Siegel 1983; MacIntyre and Pollock 1983; Klopogge and Akkerman 1984). These responses appear to involve one or more G proteins (Houslay, Bojanic et al. 1986; Hwang, Lam et al. 1986). When platelets are stimulated, PAF will accumulate on the membrane surface and works as a juxtacrine lipid mediator to start various thrombotic events (Ninio, Leyravaud et al. 1991; Zhou, Javors et al. 1992). Exogenous recombinant PAF acetylhydrolase (Ostrovsky, King et al. 1998) or PAF receptor antagonists (Weber and Springer 1997) demonstrated that NF- κ B translocates to the nucleus assisted by PAF and alters gene expression (Weyrich, McIntyre et al. 1995). These effects of PAF are generally accepted as acting through a single, unique and specific receptor, the PAF receptor (Ishii and

Shimizu 2000), which was identified taking the advantage of the development of PAF antagonists and was first clone by Honda (Honda, Nakamura et al. 1991).

1.4.3. Platelet -activating factor acetylhydrolases (PAF-AHs)

The acetyl group of PAF can be hydrolyzed by a family of enzymes called PAF-acetylhydrolases. In general there are two categories of platelet-activating factor acetylhydrolases (PAFAH): plasma or secreted type, and intracellular or non-secreted type. Macrophages are the major source of PAFAH, which has same properties to plasma form PAFAH (Stafforini, Elstad et al. 1990). Another work confirmed this conclusion. By using northern blot analysis, mRNA of PAF acetylhydrolase was found expressed in lymphatic tissues, such as tonsil, thymus, and spleen (Tjoelker, Wilder et al. 1995). It was reported that most plasma PAFAH is associated with low-density lipoproteins (LDLs) and small part with high-density lipoproteins (HDLs) (Stremmler, Stafforini et al. 1991) (Stafforini, McIntyre et al. 1987). Specific subtypes of HDL (very high-density lipoprotein-1 or VHDL-1) and LDL (small, dense LDL or LDL-5) carry most of PAFAH activity in human plasma (Tselepis, Dentan et al. 1995). However, the activities of plasma PAFAH associated with lipoproteins are different. HDL activity hydrolyzes PAF much slower than LDL activity (Stafforini, Carter et al. 1989). It was explained as that activity of PAF acetylhydrolase depends on particle concentrations of lipoproteins associated with PAFAH (Blencowe, Hermetter et al. 1995). It has been established that PAFAH also accept other physiological active molecules, e.g. fragmented oxidative phospholipids, as its substrates (Stafforini, Prescott et al. 1996). Based on enzymatic activity categorization,

PAF acetylhydrolases belong to group VII of the phospholipases A₂ (PLA₂) super family (Dennis 1997). The plasma type PAFAH hydrolyzes phospholipids with Sn-2 acyl chains with intermediate length including PAF, but only if they are oxidized including esterified isoprostanes (Stremmler, Stafforini et al. 1991). Isoform II PAF acetylhydrolase, one of the intracellular types, accepts PAF and phospholipids containing longer Sn-2 acyl residues and is well characterized as an enzyme susceptible to oxidative stress (Hattori, Hattori et al. 1995).

Type I PAFAH, a special intracellular type of PAFAH and a unique serine esterase (Hattori, Adachi et al. 1994; Ho, Swenson et al. 1997), unrelated to the other two types of the group VII PLA₂ family (Six and Dennis 2000), is very specific for the Sn-2 acetyl group of phospholipids, and has less sequence homology with any other two types of PAFAH (Arai, Koizumi et al. 2002). Interestingly, tertiary structure of it resembles that of G-protein (Ho, Swenson et al. 1997).

Type I PAF acetylhydrolase, one of the intracellular PAFAH, was initially purified from bovine brain (Hattori, Arai et al. 1993). PAF hydrolase from red blood cells was purified and found the PAFAH1B2 and PAFAH1B3 type I PAF acetylhydrolase abundantly express (Karasawa, Shirakura et al. 2005). This low molecular weight of the enzyme purified from red blood cells was found to be sensitive to inhibitors, such as sodium fluoride, DTNB, and diisopropylfluorophosphonate (Stafforini, Rollins et al. 1993). Type I PAFAH is

expressed in most human tissues. However, its biological functions have not been defined.

The type I PAFAH is a trimmer---a heterodimer of catalytic subunits, PAFAH1B3 and PAFAH1B2, with a non-catalytic LIS1 subunit. PAFAH1B2 was characterized to be a specific serine esterase with an active site at ser47 residue that had some homology to PAF receptor (Hattori, Adachi et al. 1994). PAFAH1B3 is another catalytic subunit which plays important role in brain development (Nothwang, Kim et al. 2001). PAF is believed to be the physiological substrate for type I PAFAH, because PAF hydrolyzing activity was used to purify type I PAFAH. Anti-apoptotic action of PAFAH1B2 has been shown (Bonin, Ryan et al. 2004). Animal studies showed PAFAH1B involved in spermatogenesis (Koizumi, Yamaguchi et al. 2003; Yan, Assadi et al. 2003) and neuronal migration (Manya, Aoki et al. 1998). The real physiological function of this enzyme is still not clear.

1.5. Aspirin resistance

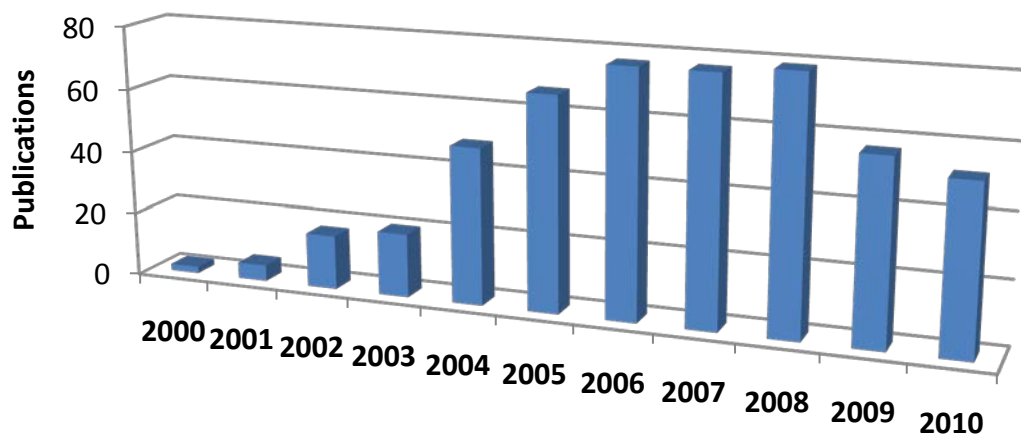


Figure 5. Scientific publications on aspirin resistance.

In terms of both efficacy and safety, patients have considerable heterogeneity in their responses to aspirin, a worldwide drug. If patients on aspirin clinically are present as treatment failure although taking the medicine at effective dosing (75–325mg/day), it is frequently and specially named “aspirin resistance”, which is of more clinical attention recently (Figure 5). Aspirin resistance is defined generally of two types, but they may have overlap to each other. “Laboratory aspirin resistance” is regarded as aspirin has partial to no effect on suppressing platelet activation *in vitro* measured by instruments such as a platelet aggregometer; or as “clinical aspirin resistance” which is defined as aspirin failing to protect patients from cardiovascular events, which is also called as “treatment failure” (Tantry, Mahla et al. 2009). Platelets can be stimulated by numerous of agonists through several pathways, so it is not surprising for aspirin resistance. Anti-platelet drugs are usually designed to block a certain activating pathway, which limits their effects on complicated thrombotic

diseases due to so many activating pathways exist. That is why clinically using more than one drug to treat patients. And another problem makes aspirin resistance more unpredictable. That is lacking universal standard methods to evaluate aspirin resistance, so the reports of aspirin resistance range from 0.4% (Tantry, Bliden et al. 2005) to as high as 70%. The most common reason believed of aspirin resistance is noncompliance (Schwartz, Schwartz et al. 2005). Other possible reasons include inadequate dosing (Roth and Calverley 1994), duration of other medication or therapy (Pulcinelli, Pignatelli et al. 2004), smoking (Macchi, Christiaens et al. 2002), variable reactions of individuals in aspirin pharmacodynamics and pharmacokinetics, such as absorption, bioavailability, metabolism, and excretion (Williams, Mutch et al. 1989; Akopov, Grigorian et al. 1992; Pappas, Westengard et al. 1994), alternative pathways of TxA₂ production (Macclouf, Folco et al. 1998; Weber, Zimmermann et al. 1999), and TxA₂ independent platelet activation pathways (Santos, Moscardo et al. 2000) have been also proposed. Also under disease conditions such as diabetes (de Gaetano 2004) and hypercholesterolemia (Szczeklik, Musial et al. 2002), the anti-platelet effect of aspirin are impaired.

However, the incidence of “aspirin resistance” may not really be increasing. Treatment failures with drugs are common phenomenon, e.g. clopidogrel resistance (Wiviott and Antman 2004). The large inter-individual variability may suggest that there are more factors affecting aspirin treatment leading to treatment failures (Schrör 2002). People usually confuse treatment failure with aspirin working failure, especially when failure of clinical treatment is frequently mixed up with a failure of aspirin to work pharmacologically (Hennekens, Schrör et al. 2004). The other question is that there is no universal standard or method to define aspirin resistance,

neither in clinical nor in the laboratory. Responses of patients with cardiovascular or cerebrovascular disease to aspirin are capricious. Same group of patients responded well to aspirin in one study, but they were found not to respond to aspirin in other studies (Helgason, Hoff et al. 1993; Helgason, Tortorice et al. 1993; Helgason, Bolin et al. 1994; Buchanan and Brister 1995; Mueller, Salat et al. 1997) (Weksler, Kent et al. 1985; Berglund and Wallentin 1991). Unfortunately, the term “resistance” has been used in articles because of its widespread acceptance, although people do not know what aspirin resistance really is. Aspirin resistance should be related to impaired aspirin effects. So aspirin resistance is classified into three distinct types (Figure 6) by biology and biochemistry methods (Weber, Przytulski et al. 2002). The first is pharmacokinetic resistance. It means that aspirin works only *in vitro* but not *in vivo*. Platelet activation is not inhibited by oral aspirin administration. However, when treating isolated platelets from the same individual with same amount of aspirin *in vitro*, both TxA₂ formation and platelet aggregation are effectively inhibited. This indicates no treatment amount of aspirin gets into platelets, namely majority of aspirin is metabolized before getting in platelets. The second is pharmacodynamic resistance. In this type of resistance, it is more like type I, but even *in vitro* the same amount of aspirin only partially inhibits platelet activation and TxA₂ production. However, by increasing aspirin dosage, inhibition can be completed. This suggests the sensitivity of targets of platelets to aspirin is impaired for some reasons. The last one is called pseudoresistance. This means that aspirin does work efficiently, because it blocks TxA₂ formation completely. However, platelets are activated normally without any

suppression. This is not a true resistance of aspirin. It is because TxA₂ independent pathways involves in platelet activation. From these three reasons for impairment of anti-platelet function of aspirin we may conclude as follow: (i) aspirin cannot reach the active site in sufficient amounts; (ii) the platelet activation factors are not sensitive to aspirin because the cellular or molecular targets of aspirin are impaired, and (iii) platelets are stimulated by other pathways which by pass aspirin targets (Schrör 2009).

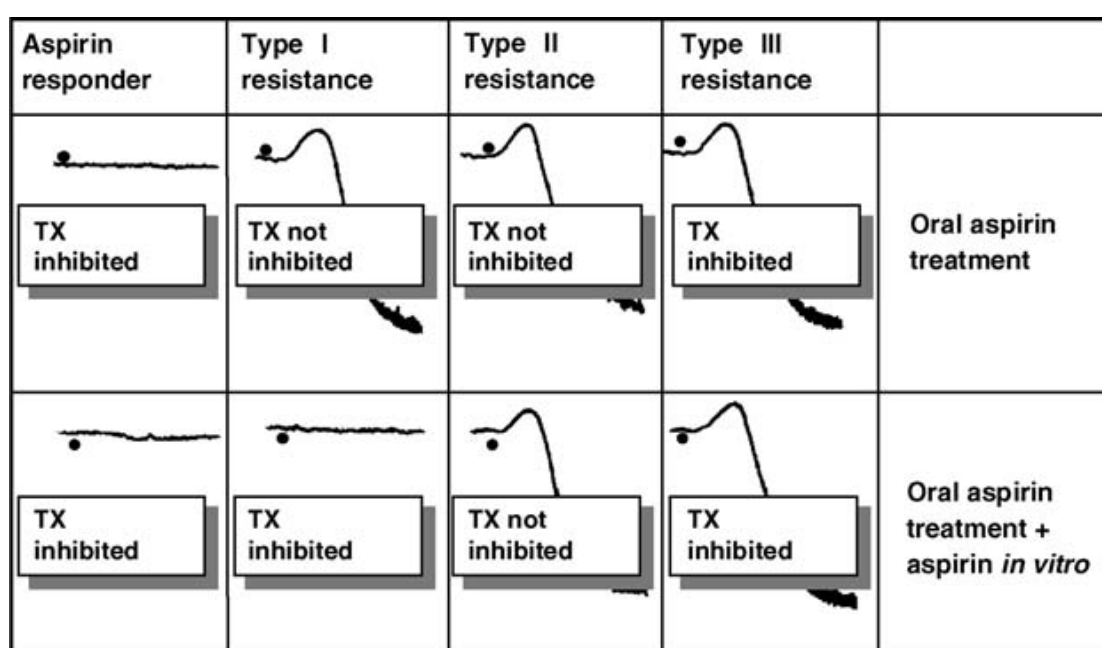


Figure 6. Types of aspirin resistance (Weber, Przytulski et al. 2002).

There are several platelet activation pathways which bypass the primary target of aspirin, namely COX-1. However, these situations may be not that important, because most of the time collagen stimulated platelet activation is prevalent in CVD, which usually occurs after the endothelium damaged. The sensitivity of aspirin target, COX-1, due to genetic factors has been examined, but effects of mutation or polymorphisms in aspirin's active site are controversial (Maree, Curtin et al. 2005; Lepantalo, Mikkelsen et al. 2006; Takahashi, Ushida et al. 2008). Only the

bioavailability reason is more common and basic problem which needs to be considered for all drugs. As previously showed aspirin is quickly deacetylated and loses its anti-platelet function in about 20 minutes in systemic circulation (Figure 7). Blood is the major place where about 70% of aspirin is inactivated. Thus, aspirin hydrolase is very important in controlling aspirin's survival or half-life in circulation, but identities of aspirin hydrolases in human blood are still undefined.

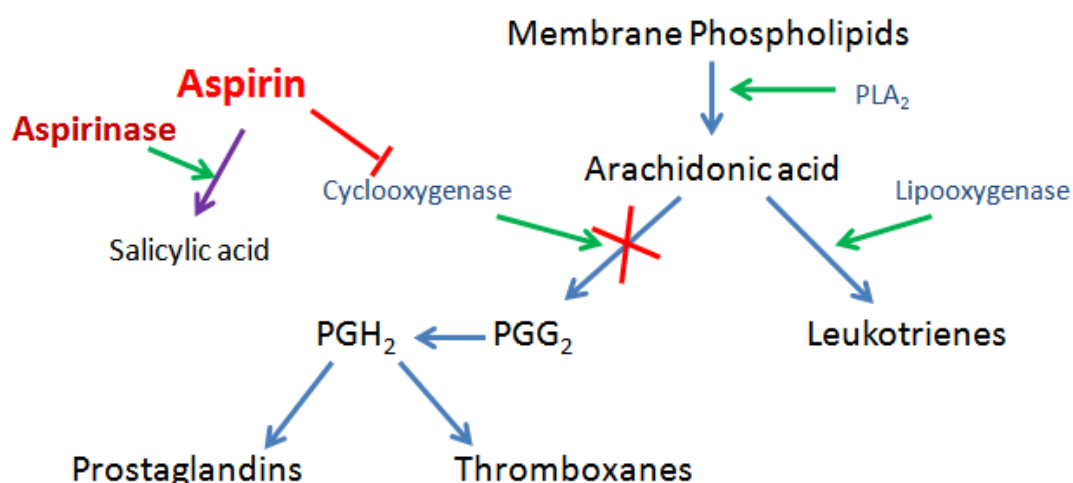


Figure 7. The mechanism of aspirin's anti-platelet function. Arachidonic acid released from membrane by phospholipase A₂ is oxidized by cyclooxygenase-1 to PGG₂, which further modified by different enzymes in individual tissues to different products. Aspirin inhibits cyclooxygenase-1 irreversibly by acetylating 530-serine residue.

1.6. Aspirin esterase

Aspirin esterases are important in controlling aspirin hydrolysis. They include several enzymes which can remove the acetyl group of aspirin. Enzymatic hydrolysis of aspirin starts in the gastrointestinal mucosa after being given orally (Levy 1978; Spenney 1978) and continues in liver and the portal vein (Levy 1979; Williams, Mutch et al. 1989), which is called the first pass effect. After the first pass effect by liver and gut, about 70% of aspirin reaches blood and it is metabolized to salicylic acid by serum esterases (Rainsford, Ford et al. 1980; Seymour, Williams et al. 1984) and by erythrocytes (Costello and Green 1983; Costello, Caruana et al. 1984). So blood is the major place to hydrolyze aspirin. The half-life of aspirin in circulation is around 15–20 min (Levy 1965; Rowland, Riegelman et al. 1972; Shen, Wanwimolruk et al. 1991), which is mainly controlled by blood esterases. The esterases classification is a hard work, so there are different classifications of esterases. In 1953, by using different organophosphorous esters as substrates, Aldridge classified esterases as A, B, or C groups (Aldridge 1953). By involving inhibitors in the classification besides substrates, in 1961 Augustinsson classified esterases as cholinesterases, aryl esterases and carboxylesterases (Augustinsson 1961; Ali and Kaur 1983). Human plasma has only cholinesterases and aryl esterases, but no detectable carboxylesterases (Li, Sedlacek et al. 2005). Plasma albumin is also considered as an esterase (Kurono, Kondo et al. 1983; Sakurai, Ma et al. 2004; Yang, Bian et al. 2007; Lockridge, Xue et al. 2008). But this issue is controversial, in 2001, Chapuis showed that esterase-like activity of serum albumin was probably due to butyrylcholinesterase contamination (Chapuis, Bruhlmann et al. 2001). Until now there are three esterases claimed to be present in human plasma that hydrolyze aspirin. They are butyrylcholinesterase (Morikawa, Inoue et al. 1979; Masson, Froment et al. 1998), paraoxonase-1

(Santanam and Parthasarathy 2007) and albumin (Morikawa, Inoue et al. 1979; Liyasova, Schopfer et al. 2009). But this is not widely accepted, and people still use the term aspirin esterase to indicate any enzyme that hydrolyses aspirin (Adebayo, Williams et al. 2007).

Butyrylcholinesterase: Butyrylcholinesterase (BChE), also called plasma, serum or pseudo cholinesterase, is a tetrameric glycoprotein with a molecular mass of 342 kDa. BChE gene is located on chromosome 3 at long arm, fragment 26. It is synthesized and secreted primarily from the liver (Lockridge, Adkins et al. 1987) and finally distributed in mucosa of the intestines, blood and the white matter of the central nervous system. Each identical subunit of BChE has an active catalytic site (Bergmann and Wurzel 1954). The physiological function of BChE is largely unknown. Human plasma aspirin hydrolytic activity and BChE activity are skewed and positively correlated (Adebayo, Williams et al. 2007). BChE requires Ca^{2+} to maximize its activity when catalyzing hydrolysis of its known substrates. BChE can hydrolyze aspirin, although it is different from other substrates in kinetics (La Du 1971). There are several kinds of human BChE deficiencies known due to different genetic reasons, including FS117 first described by Liddell et al. (Liddell, Lehmann et al. 1962). It may have a homozygous frequency of 1:100,000. This mutation caused a reading frame change at the first base pair and created a stop codon (TGA) at position 129aa. The changes would make a product of 22% of the mature protein (601aa) and are upstream of the serine-198 active center, which makes no detectable BChE activity in plasma. Clinically, these individuals appear to be normal.

Paraoxonase (PON): paraoxonases, a family of three isomers, PON1, PON2 and PON3, are a group of enzymes having organophosphates hydrolytic activity. The

genes coding PON are located together on 7q21.3-q22.1 (Primo-Parmo, Sorenson et al. 1996). PON2 is intracellular and expressed widely in liver, brain, lungs, and heart, but not found in blood (Mochizuki, Scherer et al. 1998). Both PON1 and PON3 are found in blood, which may be secreted from liver (Mackness, Hallam et al. 1985; Reddy, Wadleigh et al. 2001), but they have different substrate specificities. PON1 is well investigated member in the paraoxonase family. It is a calcium dependent hydrolase with the ability to recognize a broad spectrum of synthetic substrates. It is tightly bound to plasma high density lipoproteins (HDL) and exhibits anti-oxidative activities, which can protect low density lipoprotein (LDL) from being oxidized. However, some of its properties appeared to be due to PAFAH contamination that co-eluted with PON1 during purification (Marathe, Zimmerman et al. 2003; Kriska, Marathe et al. 2007). In 2007, aspirin was reported to be a substrate for PON1 (Santanam and Parthasarathy 2007).

The identities of the enzymes in blood that actually hydrolyze aspirin have not been elucidated despite they all appear to hydrolyze aspirin and to affect aspirin levels, although these enzymes may be factors in defining the poorly documented syndrome of aspirin resistance (Fitzgerald and Maree 2007; Patrono and Rocca 2007; Lev 2009).

CHAPTER II

2. MATERIALS and METHODS

2.1.1. Commercially available reagents:

Chemicals: Aspirin, Salicylic acid, o-Anisic acid, Brilliant Blue R-250, Formic acid, Anthranilic acid, Acetaminophen, 5, 5'-Dithiobis (2-nitrobenzoic acid), sephadex G-100, Blue Dextran, Acetyl- β -methylcholine chloride, Adenosine 5'-triphosphate, 4-Methylumbelliferyl acetate, Butyrylcholine chloride, S-Butyrylthiocholine iodide, Adenosine 5'-triphosphate, periodate oxidized sodium salt, Adenosine 5'-triphosphate immobilized on agrose 4B, cell lysis buffer 10 \times (Cell Signaling).

2.1.2. Antibodies:

BChE (C-18), BChE (N-15), AGP1 (27A1), APEH (N-18), APEH (C-15), CD39 (BU61), and β -actin (Santa Cruz, CA); anti-PAFAH1B2 (16-30) and PAFAH1B3 (SAB1100319, Sigma)

2.2. Methods

2.2.1. Aspirin hydrolytic activity assay by Reverse Phase High Performance Liquid Chromatography

Aspirin (Sigma, MA) 18mg is dissolved in 1ml of methanol. 10 μ l of aspirin solution is transferred into each tube and dried under N₂. Enzyme source and buffer is added to bring the volume to 200 μ l. Incubation is performed at 37°C for 1 or 2 hours as stated, depending on the activity of the enzyme source. After incubation the reaction is stopped by adding 0.1% formic acid (200 μ l) and aspirin and salicylic acid are twice extracted by a mixture of ethyl acetate and ether (4:1, v/v) 1ml. The supernatant is then blown dry under N₂ before being reconstituted into 500 μ l HPLC mobile phase and 100 μ l being injected. Then on to a 4.6 \times 150mm, 5 μ m, Eclipse XDB-C8 column (ZORBAX, Agilent), mobile phase was methanol: water (40/60, 1% acetic acid, v/v) at a flow rate of 0.8ml/min. Aspirin and salicylic acid display retention times around 7 and 13 minutes (Figure 9). Standard curve was plotted to estimate salicylic acid produced by aspirin hydrolysis (Figure 8).

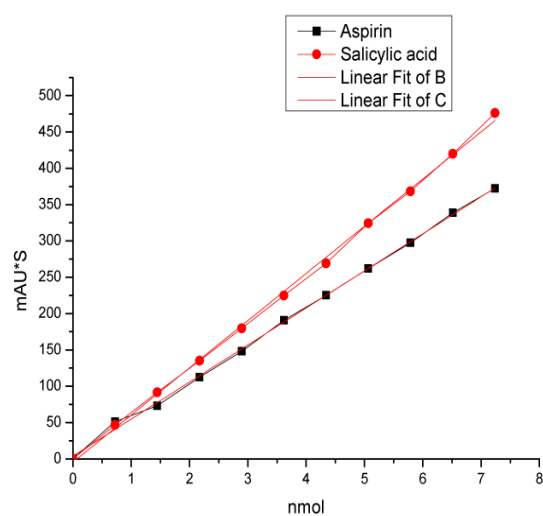


Figure 8. Linear relationship between under curve area and the amount of Aspirin and Salicylic acid. Different amount of Aspirin and Salicylic acid (10, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900ng) were injected into HPLC. Signals were detected at 280nm.

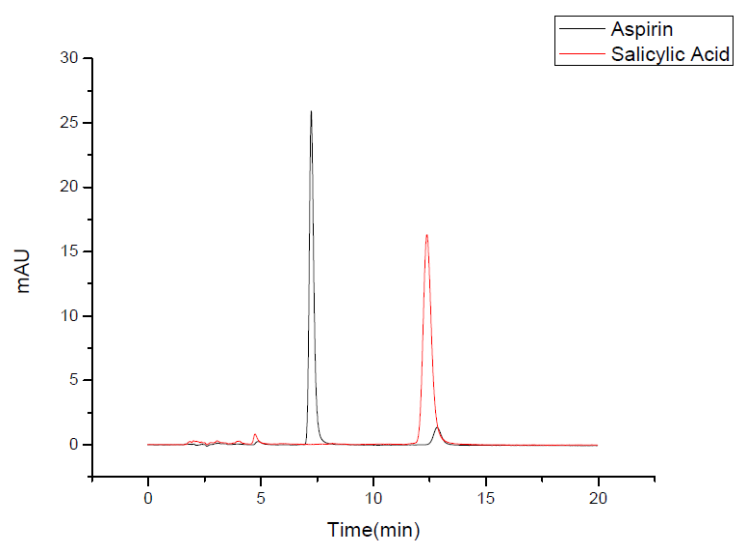


Figure 9. Separation of Aspirin and Salicylic acid by RP-HPLC. The retention time of Aspirin is around 7 minutes; the retention time of Salicylic acid is around 13 minutes.

2.2.2. High-throughput Aspirin hydrolytic activity assay by Reversed Phase High Performance Liquid Chromatography

In order to make quantification of salicylic acid suitable for a high throughput measurement of aspirin hydrolysis, I developed a faster assay using 96 well round bottom plates. Here, the total reaction volume was 50 μ l, which contained 2mM aspirin. This was incubated with 10 μ l enzyme source at 37°C for 2 or 3 hours. After incubation the reaction was stopped and proteins were precipitated by adding 150 μ l acetonitrile containing 0.1% of formic acid. The plate was centrifuged at 4,000 \times g at 4°C for 20 min before 10 μ l of the supernatant was injected onto the column of an Agilent 1100 HPLC (2 \times 150mm, 5 μ m; ODS, Phenomenex). The mobile phase was acetonitrile: water (40/60, v/v) containing 0.1% formic acid and flow rate was 0.4ml/min.

Acetaminophen was used as an internal standard (Figure 10), allowing calculations to analyze aspirin hydrolysis according to under curve area. The concentration of salicylic acid defined by $\frac{HPLCvalue \times 200\mu l \times dilutionfactor}{hours \times 138.12}$ was used to calculate hydrolyzed aspirin per hour.

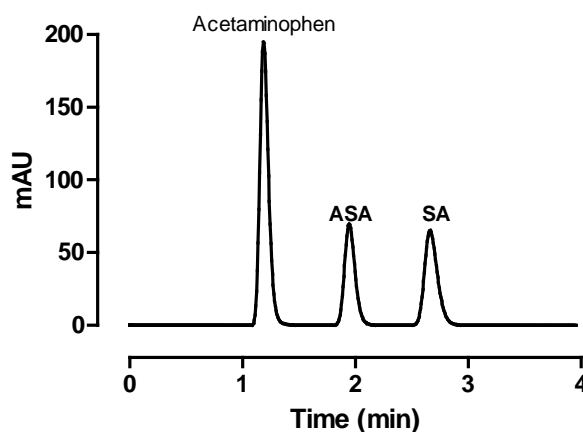


Figure 10. Isolation of acetaminophen, aspirin, and salicylic acid. A mixture of acetaminophen, aspirin, and salicylic acid was separated by RP-HPLC. Elution time of acetaminophen was 1.2 min; elution time of aspirin was 2 min; elution time of salicylic acid was 2.6 min.

2.2.3. BChE activity staining

This method uses butyrylthiocholine instead of butyrylcholine as a substrate of BChE, whose hydrolysis generates a thio-group the reduced to form a copper ferrocyanide colored precipitate. Briefly, 0.2 M maleic acid (180 ml, 4.18g/180ml) was adjusted to pH 6 before use and then 0.1 M sodium citrate (15 ml, 441mg/15ml) was added into the maleic acid buffer. Then 0.03 M CuSO_4 (30 ml) was added with 5 mM potassium ferricyanide (30 ml, 49.3875mg/30ml) (avoiding light and acid) before the final volume was adjusted to 300ml. Finally 2 mM butyrylthiocholine iodide (0.6 mg/ml; 171mg in 285ml) was added. Gels electrophoretically resolving proteins under native condition were incubated with staining solution under gentle shaking, allowing brown-red bands to develop (Li, Sedlacek et al. 2005) (Figure 11).

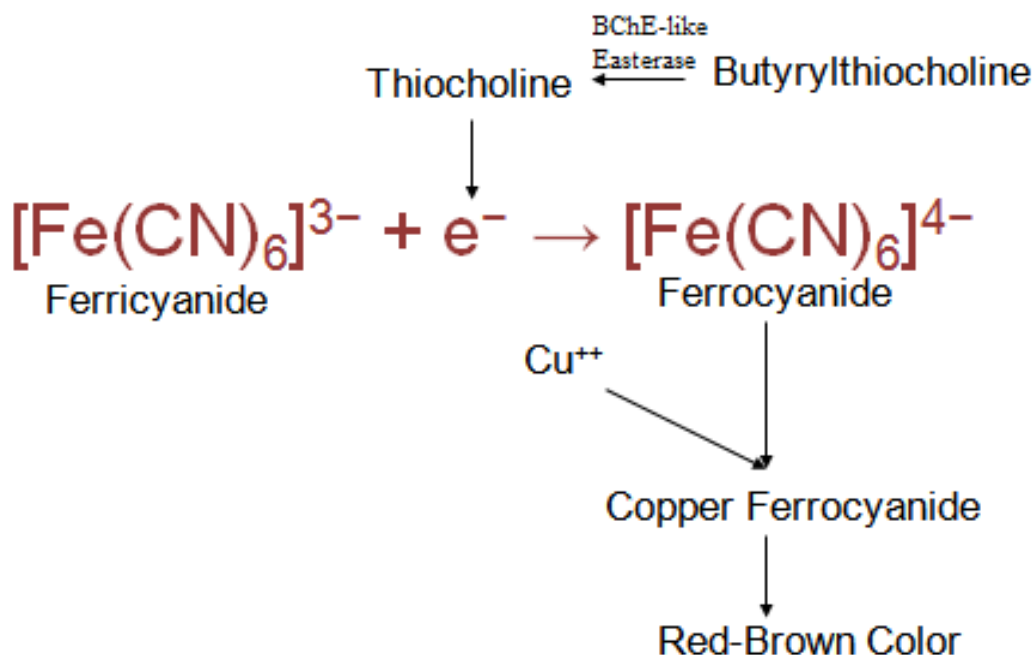


Figure 11. Reaction of BChE activity staining. Butyrylthiocholine was employed as a substrate of BChE. Product thiocholine further reacts with ferricyanide to produce ferrocyanide which reacts with copper ion to make red-brown color precipitation.

2.2.4. BChE activity assay

This method uses the Ellman reaction (Figure 12) with butyrylthiocholine as a substrate of BChE. The yellow reaction product is quantified at 412nm using both 96 well plate and 1.5ml cuvette format. A DTNB stock solution (10mM in 10×PBS, diluted 10 times before use) and 200mM butyrylthiocholine iodide were mixed just before use at a 100 to 1 ratio, respectively.

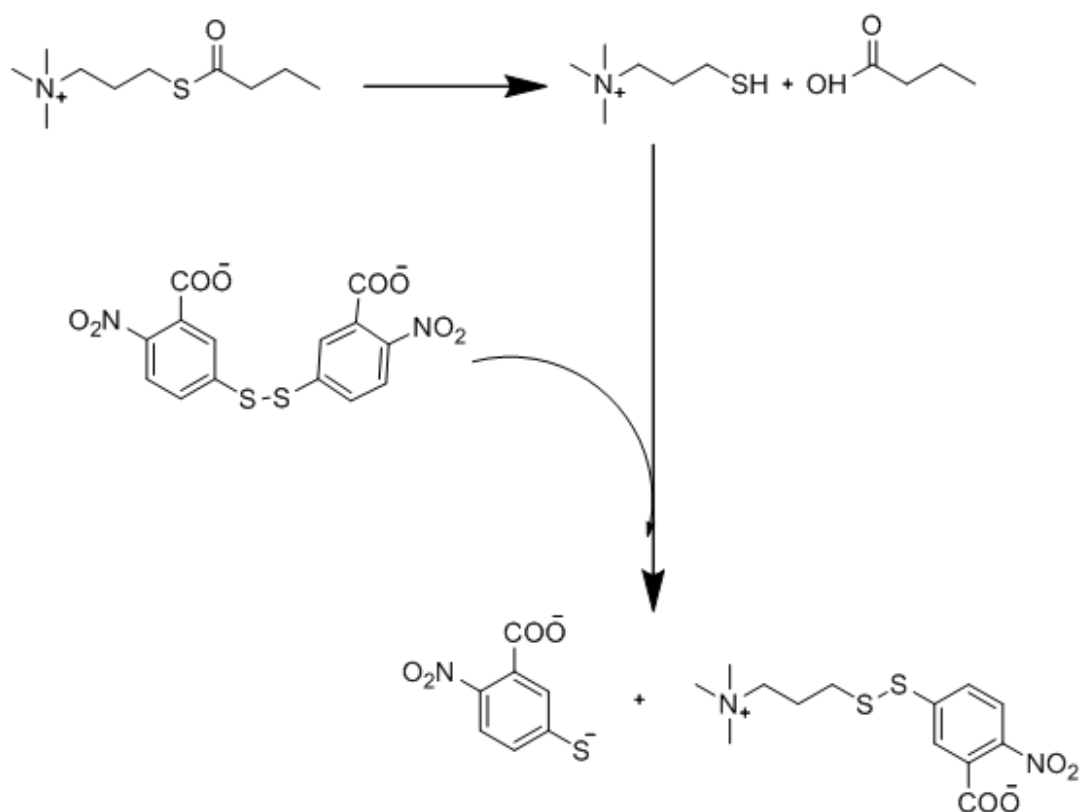


Figure 12. Ellman reaction. Thiocholine produced from butyrylthiocholine reacts with 5, 5'-dithiobis(2-nitrobenzoate) to form a yellow compound which can be detected and quantified at 412nm.

2.2.5. Isolation of HDL, LDL, and VLDL

200ml blood was drawn from a normal individual following a protocol approved by the Cleveland Clinic Institutional Review Board. Blood in 50ml aliquots was added into EDTA containing centrifuge tubes (500 μ l of 0.25M EDTA) and centrifuged 20 minutes at 4°C at 2,000 \times g. Plasma (9ml) was mixed with 4.5 g KBr and rocked for 15 minutes for complete dissolution before 27 ml saline solution was added to each of 8 ultracentrifuge tubes. The KBr plasma mixture was added into the bottom of each ultracentrifuge tube before the tubes were sealed using a Beckman hot seal apparatus. The sealed tubes were centrifuged at 50,000 \times g in Ti50 rotor at 4°C for

3 hours. After centrifugation, fractionation was performed by using P-3 Pharmacia peristaltic pump connected to a Frac-100 and/or to Gilson fraction collector to collect aliquot of 1 ml per tube (Figure 13). The specific gravity of the fractions was measured. Fractions containing LDL are those having a density of less than 1.063 g/ml, but more than 1.019 g/ml.

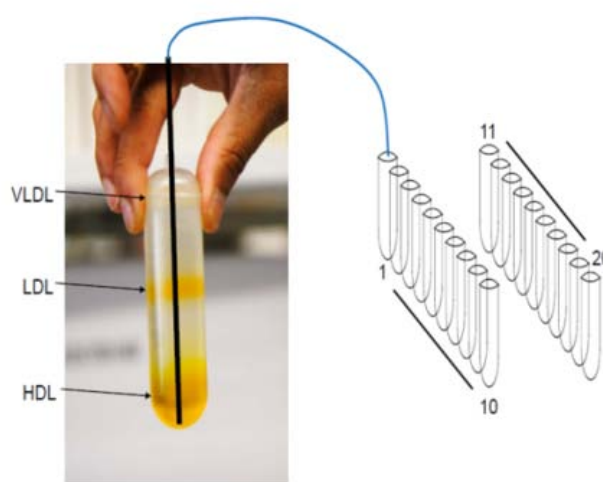


Figure 13. HDL, LDL, and VLDL separation by gradient density ultracentrifugation. HDL, LDL, and VLDL were separated by gradient density ultracentrifugation and were fractionated to 18 fractions from HDL to VLDL.

2.2.6. PAF-AH activity assay

Substrate and column preparation: PAF (41.84 μ l) was taken from stock of 5 mg/ml in methanol, 400 nmol unlabeled PAF. [3 H]PAF (9 μ l, 10 μ Ci/nmol) was mixed with the unlabeled PAF and dried. The mixed PAF was suspended in 4 ml of HEPES buffer and frozen as 500 μ l aliquots. Octadecylsilica gel cartridges were washed with 3 ml CHCl_3 : methanol (1:2, v/v), then 3 ml 95% ethanol and finally with 3 ml H_2O .

Assay: A mixture of 10 μ l enzyme source with 5 μ l 400mM DTT and 40 μ l of PAF substrate were incubated at 37°C for 15 minutes. 10 M acetic acid (50 μ l) was

added to stop the reactions and 750µl of 0.1 M sodium acetate. The released acetate (Figure 14) was isolated by passing through pre-washed Baker octadecylsilica cartridges (1ml, J.T. Baker Chemical Co.) before collecting the effluent in a scintillation vial containing 15 ml of scintillation cocktail (Ecolite™) for quantification by a β-scintillation counting.

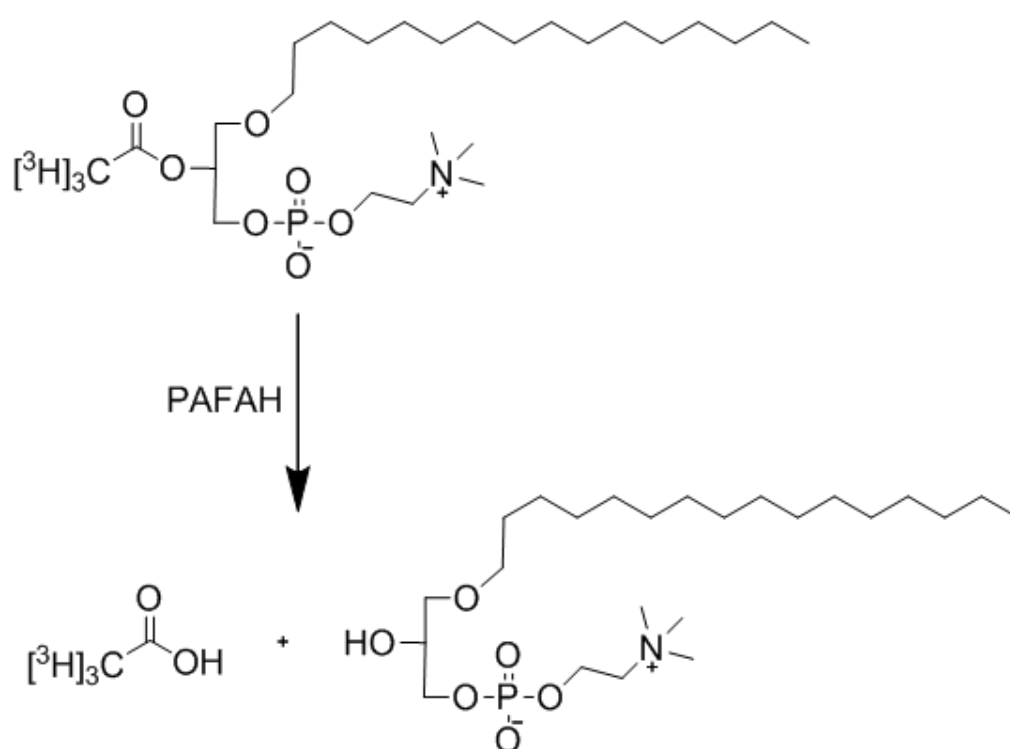


Figure 14. PAF-AH hydrolyzes the sn-2 acetyl group of PAF. Cleaved $[^3\text{H}]$ labeled acetate was detected by scintillation counting to monitor hydrolyzed PAF.

2.2.7. Western Blotting

Samples were prepared using 2× loading dye containing 5% of β-Mercaptoethanol before SDS-PAGE was performed to separate the proteins. These were then transferred onto PVDF membranes and then incubated with 5% nonfat milk for 1 hour. Diluted primary antibodies (1:200-1:1000 according to the manufactory's instruction) were incubated with the membrane with rocking for 1 hour and followed by three TBS washes. Species specific secondary antibodies (HRP-conjugated, 1:10,000) were used to ligate the primary antibodies. Enzymatic Chemiluminescence (ECL) reagents (GE healthcare) and x-film were used to visualize the phosphorescence.

2.2.8. Protein quantification

Absolute quantification of protein concentration or mass is a required and routine step to estimate protein recovery at different stages during purification of proteins, to measure specific activity of enzyme, and to prepare known amount of protein for biophysical, functional and analytical analyses. Absolute measurements of protein require calibrated sets of external standards containing known amounts of stereotypic proteins. However, when comparing the relative amounts of proteins among different samples, external standards are not needed. There are four methods that are commonly used to quantify proteins, which were used more or less in my project, because each of these methods has its advantages and limitations. They are absorption of UV light by side chains and / or peptide bonds of the protein;

chromogenic reactions involving complexes formed under alkaline conditions between the protein and cuprous ions (BCA); binding of a chromophore to the protein (Bradford); dot-blotting and staining.

Advantages and disadvantages of each method are concluded as followed. UV method can be performed directly on the samples, but in affectedly strong interferences by nucleic acids, buffer salts, and detergents; BCA (bicinchoninic acid) is compatible with detergents and adaptable for microtiter plate assay (Redinbaugh and Turley 1986), but it is interfered with by reducing agents, chelators, and lipids (Wessel and Flugge 1984; Morton and Evans 1992); Coomassie dye binding method is compatible with reducing agents and adaptable for microtiter plate assay (Redinbaugh and Campbell 1985), but not compatible with detergents (Morton and Evans 1992).

Proteins from plasma or erythrocyte lysates were dialyzed against 50mM Tris-HCl buffer, pH 7.2 at a 100 fold volume at 4°C overnight 3 times. Some measurements were performed without protein dialysis. Methods were chosen as stated using instructions of manufacturer.

2.2.9. Platelet aggregation

Platelet activation was measured indirectly by measuring aggregation optically in an aggregometer (CHRONO-LOG, model 700). Washed platelets (2×10^8) were stirred at 600 rpm at 37°C before stimulation with 5mg/ml collagen. Slope and

percentage of aggregation were recorded by monitoring light transmission compared to buffer control.

2.2.10. Cell culture and Overexpression

The human embryonic kidney 293 cell line (HEK 293) from the American Type Culture Collection (ATCC) was grown in Dulbecco's Modified Eagle Medium (DMEM) (Cleveland Clinic Foundation Medium Core Facility) supplemented with 10% fetal calf serum (Hyclone), and penicillin (100U/mL) and streptomycin (100µg/mL) in a humidified CO₂ (5%) atmosphere at 37°C. HEK 293 cells (1×10⁵ cells/well) were plated in 6-well plates. After 24 hours the cells were transfected with DNA plasmids of human platelet-activating factor acetylhydrolase 1B with a CMV promoter using lipofectamineTM 2000 (Invitrogen, Carlsbad, CA) that followed the manufacturer's instructions. After 32 hours cells were collected and washed twice by 1× PBS buffer.

2.2.11. Protein purification

Cell Isolation: Human erythrocytes were isolated from freshly drawn human blood (400 ml) from healthy volunteers. The blood was collected in EDTA (25 mM) and centrifuged at 2,000× g for 20 min. The supernatant was discarded, and the blood cells were suspended in saline solution (600 ml) and centrifuged as above. Recovered erythrocytes were washed twice more to remove contaminating plasma. All chromatography was carried out at 4 °C.

DEAE chromatography: Erythrocytes (100 ml) were lysed hypotonicly by 100 ml 0.2% NaCl. Membrane debris was isolated by ultracentrifugation at 10,000×g for 1

hour at 4°C. Supernatants (200 ml) of erythrocyte lysates were diluted by to 1L in 50mM Tris-HCl (pH 7.4) containing 1 mM EDTA and loaded onto 500 ml DEAE SepharoseTM FF column. The column was conditioned with equilibrating buffer (50 mM Tris-HCl pH 7.4) until virtually no protein eluted from the resin (1-1.5 liters of buffer). This step removed most of the hemoglobin, which is one of the major proteins in this preparation. The aspirin hydrolytic activity was eluted from the gel by 0-0.5 M linear gradient of sodium chloride in 50 mM Tris-HCl (pH 7.4). Fractions (8ml) were collected and assayed for protein content and aspirin hydrolytic activity. The major aspirin hydrolytic activity was detected in fraction 34 to fraction 44. These fractions were pooled and concentrated to 20 ml using Pierce[®] concentrators (Thermo scientific). The concentrated preparation (5 ml) was diluted to 100 ml with 50 mM Tris-HCl (pH 7.4) and passed through a second DEAE column (50 ml) and eluted with linear gradient sodium chloride solution (0-0.1M) buffered by 50 mM Tris-HCl (pH 7.4). Fractions (4 ml) were collected and assayed for protein content and aspirin hydrolytic activity. Most of the aspirin hydrolytic activity was again present in fraction 35 to fraction 43. Fractions with aspirin hydrolytic activity were pooled and concentrated to 5 ml using Pierce[®] concentrators (Thermo scientific).

HiTrap Q chromatography: Concentrated preparations from DEAE chromatography (5 ml) were diluted to 60 ml and loaded onto a 5 ml HiTrap Q column conditioned by 50 mM Tris-HCl (pH 7.4) and eluted with a 0-0.1M linear gradient NaCl. Fractions (1 ml) were collected and measured for protein content and aspirin hydrolytic activity. The major aspirin hydrolytic activity was in fraction 40 to

fraction 50. After measuring activity, fractions with aspirin hydrolytic activity were pooled and concentrated to 1 ml using Pierce[®] concentrators (Thermo Scientific).

Sephacryl S-200 chromatography: The pooled, concentrated fractions from the column HiTrap Q column were loaded on a Sephacryl S-200 column (1 × 60 cm) conditioned in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. Collected fractions (1 ml) were assayed for protein content and aspirin hydrolytic activity before pooling and concentrating active fractions.

2.2.12. Mass spectrometry

Purified protein was further separated by SDS-PAGE. The bands were carefully cut from the Coomassie stained gel with a special cleaned cutter and then destained in ethanol (50%) and acetic acid (5%). The destained bands were dehydrated in acetonitrile, dried in a Speed-vac, and digested with trypsin. Briefly, 5 µL of 10 ng/µL trypsin is added in 50 mM ammonium bicarbonate and incubated with dried bands overnight at room temperature and followed by peptides extraction by adding two aliquots of 30 µL acetonitrile (50%) containing formic acid (5%) (Gandhi, Wang et al. 2008). The LC-MS used was a Finnigan LTQ linear ion trap mass spectrometer system. The HPLC column was a self-packed 9 cm × 75 µm id Phenomenex Jupiter C18 reversed-phase capillary column. 10µL of the extracted peptides mixture were injected onto the column and then was eluted by a mobile phase of acetonitrile/0.1% formic acid gradiently at a 0.25 µL/min flow rate, which was then introduced into the on-line mass spectrometer. The data were searched

against the NCBI non-redundant database via Mascot using a human taxonomy filter and verified by manual interpretation.

2.2.13. Enzyme Kinetics:

Aspirin concentrations were varied over a range of 0.25 mM to 8 mM with incubation for 1 hour at 37°C. Some experiments were incubated with aspirin in the presence of platelet-activating factor as an inhibitor. Experiments were triplicate and mean was used to further analysis. Data were fitted using the prism 4 software to obtain kinetic parameters.

CHAPTER III

3. CHARACTERIZATION AND IDENTIFICATION OF HUMAN PLASMA

ASPIRIN HYDROLASES

3.1. Introduction

Aspirin, the first synthesized drug, is commonly used as an analgesic, anti-pyretic, anti-inflammatory, and anti-platelet drug in the world and it is consumed over 100 billion tablets each year (Warner and Mitchell 2002). Aspirin significantly reduces mortality and re-infarction in cardiovascular diseases (Awtry and Loscalzo 2000; Baigent, Blackwell et al. 2009). It is widely used as a prophylaxis reagent of cardiovascular and thrombotic diseases in a low dose (75-81mg/day) manner. This takes advantage of aspirin's anti-platelet function, which first known in 1980s.

Aspirin is a derivative of salicylic acid by acetylation and its pharmacological and molecular structure (Byrn and Siew 1981; Kim and Machida 1986) characters resemble those of salicylic acid. But the reactive acetyl group gives aspirin specific biological activities comparing to salicylic acid through the nonselective acetylation of proteins and DNA (Pinckard, Hawkins et al. 1968). Studies have shown that a lot of proteins, nuclear acids, and lipids can be acetylated by aspirin *in vivo* and *in vitro*

(Thun, Namboodiri et al. 1991). The acetylation effect of aspirin has also been seen in albumin (Zandi and Breitner 2001), hemoglobin (Bridges, Schmidt et al. 1975), and erythrocyte membrane peptides (Fiorucci, de Lima et al. 2002), suggesting acetylation by aspirin should be nonspecific. However, the primary medical therapeutic target of aspirin in preventing CVD is showed as acetylation of Cyclooxygenase-1 in platelets and Cyclooxygenase-2 in other cells. The primary target of aspirin in platelets, Cyclooxygenase-1, is irreversibly inactivated by acetylation at its serine 530 residue (Smith, DeWitt et al. 1990) (Vane 1971). Cyclooxygenase-1 is continually synthesized in nucleated cells to substitute the inactive ones, but this will not happen in anucleate platelets. The ideally situation for anti-platelet effect of aspirin is that Cyclooxygenase-1 pathways are blocked specifically in platelets without touching that in other cells. Prostaglandin H_2 metabolized from arachidonic acid by PLA_2 in platelets is rapidly converted to the unstable endoperoxide thromboxane A_2 (TxA_2), that specifically stimulates the thromboxane A_2 (TxA_2) receptor of platelets (FitzGerald 1991). Activation of this receptor courses and enhances platelets response to stimuli by agonists including ADP and collagen, so if blocking this pathway platelet reactivity will be reduced. Thus, carefully choosing the right doses of aspirin is important to increase therapeutic effects and reduce side effects.

Aspirin is transient with a half-life of 15-20 minutes in circulation, but its inhibition of cyclooxygenase is irreversible resulting in lifelong suppression of platelet thromboxane synthesis (Needs and Brooks 1985; Higgs, Salmon et al. 1987; Awtry and Loscalzo 2000). However, certain individuals, e.g. diabetics or stroke

survivors, may not receive the full protection from CVD by low dose aspirin administration, although defining, measuring and assessing aspirin resistance is complex and incomplete (Fitzgerald and Maree 2007; Patrono and Rocca 2007; Lev 2009; Santilli, Rocca et al. 2009; Pignone and Williams 2010). As I discussed above, acetyl group is the key for aspirin in suppressing platelet function. Hydrolysis of its acetyl group will destroy its anti-platelet ability. However, aspirin hydrolysis in serum is not normally distributed (Adebayo, Williams et al. 2007) and is increased in patients with type 2 diabetes (Gresner, Dolnik et al. 2006; Kotani, Kimura et al. 2010), atherosclerosis (Akopov, Grigorian et al. 1992), aspirin-sensitive asthma or cold urticaria (Williams, Asad et al. 1987), or after surgery (Puche, Gomez-Valverde et al. 1993). Since aspirin hydrolysis *in vivo* is an enzymatic event, looking for responsible enzymes for aspirin hydrolysis will be a worthwhile way to unveil the complex.

The identity of the enzyme(s) in plasma that hydrolyzes aspirin remains unknown despite its wide use and hundred years' history. The effects of turnover on its efficacy are important, which is directly controlled by aspirin hydrolases. Aspirin hydrolase activity and butyrylcholinesterase (BChE) activity in plasma are correlated positively and their distributions are skewed (Adebayo, Williams et al. 2007). However, BChE hydrolyzes aspirin, which is different from its way of hydrolyzing butyrylcholine esters (La Du 1971). The other aspirin hydrolytic activity in plasma is still undefined, although it is believed to be albumin (O'Brien 1968). Aspirin hydrolytic activity has been found in cells where enzymes were shown to include uridine diphosphate glucuronyl transferase (Bigler, Whitton et al. 2001; Chan, Tranah

et al. 2005) and carboxylesterase 2 (Mentlein and Heymann 1984; Yamaori, Fujiyama et al. 2006). Thus, aspirin hydrolysis is not a behavior of a single enzyme that hydrolyzes aspirin.

Because aspirin has acetylation effects, which is a nonspecific process, assessing aspirin hydrolysis becomes complicated. For example, loss of [^3H]acetate from [^3H]aspirin includes non-enzymatic derivitization of acceptors such as albumin (Yang, Bian et al. 2007; Lockridge, Xue et al. 2008) and water. There are esterases with broad spectrum substrate specificity, so-called non-specific esterases, which catalyze aspirin hydrolysis. These might be the reason enzymes responsible for aspirin hydrolysis is still undefined. However, aspirin is an artificial compound and its hydrolysis by enzymes is not an evolutionarily selected trait, and so reflects the action of one or more esterases that happen to accept it as a substrate. Fortunately, there are studies which have been found enzymes as aspirin hydrolases, but still far way to go to elucidate aspirin resistance. Esterases hydrolyzing aspirin include butyrylcholinesterase, abundantly existing in plasma (Masson, Froment et al. 1998) (Adebayo, Williams et al. 2007). Most recently paraoxonase-1 (PON1) has been shown to hydrolyze aspirin and aspirin nitrate, a novel anti-inflammatory agent (Santanam and Parthasarathy 2007).

The physiologic function of plasma butyrylcholinesterase is not well defined. Its hydrolysis of organophosphates is of potential benefit after exposure to organophosphate based nerve agents (Saxena, Sun et al. 2011), and it hydrolyzes

acetylated drugs and choline-containing local anesthetics (Goodall 2004). The genotype and phenotype of butyrylcholinesterase are convoluted, which leading to the kinetics of the tetramer (Goodall 2004) are complex (Simeon-Rudolf, Reiner et al. 1999). It has several identified isomers and mutations, which makes understanding physiological role of BChE more difficult. People tried to distinguish these variations base on the dibucain number that reflects catalysis and clearance of local anesthetics (Goodall 2004). Butyrylcholinesterase hydrolyzes aspirin (Masson, Froment et al. 1998), but its actual contribution to aspirin hydrolysis in plasma remains undefined.

I directly assessed aspirin hydrolysis in human plasmas by isolating and quantifying the salicylic acid produced from aspirin by RP-HPLC to find aspirin hydrolysis in plasma among people is complicated, and highly variable. PON1 was found to not hydrolyze aspirin. Butyrylcholinesterase hydrolyzes aspirin, but BChE null plasma still hydrolyzed aspirin normally. I found significant variation exists in batch total plasma aspirinase activity and the variation of plasma aspirin hydrolytic activity is genetically encoded. Genome wide association analysis effectively correlated gene of butyrylcholinesterase with this variation, although genetic deletion was not informative. However, a non-BChE aspirinase activity with little variation exists in plasma that I show to conduct with type I PAF acetylhydrolase.

3.2. Results

3.2.1. Plasma hydrolyzes aspirin enzymatically.

Structurally, aspirin should have had the most same pharmacological properties as salicylic acid. However, after the acetyl modification, aspirin gains new functions, anti-platelet function, and promote generation of biologically active Lipoxins (Brezinski, Nesto et al. 1992). After incubation with plasma at 37°C for 2 hours, aspirin was found mostly hydrolyzed to salicylic acid (Figure 15). This is an enzymatically behavior. When I heated plasma at 75 °C for 2 hours, it totally lost its ability to hydrolyze aspirin (Figure 16). I also determined the effect of time on aspirin hydrolysis by plasma, and found it was linear (Figure 17). Since aspirin hydrolysis in plasma is an enzymatical behavior, I also examined effect of EDTA, an anti-agglutination agent, on aspirin hydrolysis. No effect of EDTA was found on aspirin hydrolysis by plasma (Figure 18).

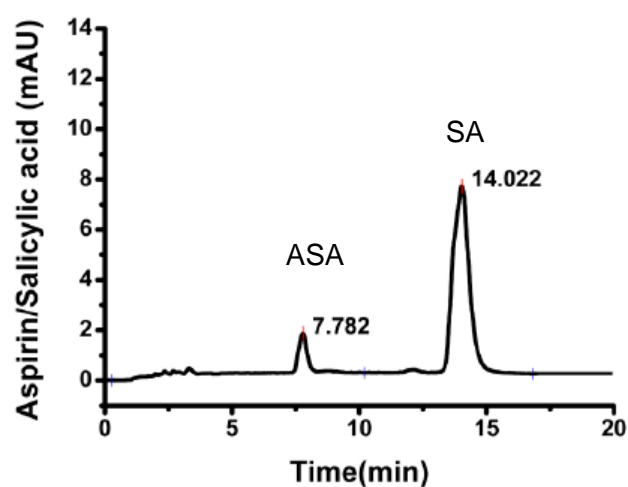


Figure 15. Human plasma hydrolyzes aspirin efficiently in 2 hours. Aspirin (10 μ g) was incubated with 100 μ l 1mM EDTA treated human plasma at 37°C for 2 hours. The reaction was stopped by adding 1% formic acid, and aspirin and salicylic acid were extracted by ethyl acetate: diethyl ether (4:1, v/v). The organic phase was separated and dried under N₂. Recovered aspirin and salicylic acid were reconstituted in 500 μ l of HPLC solvent (1% glacial acetic acid: methanol, 60:40, v/v) and 50 μ l was analyzed by RP-HPLC.

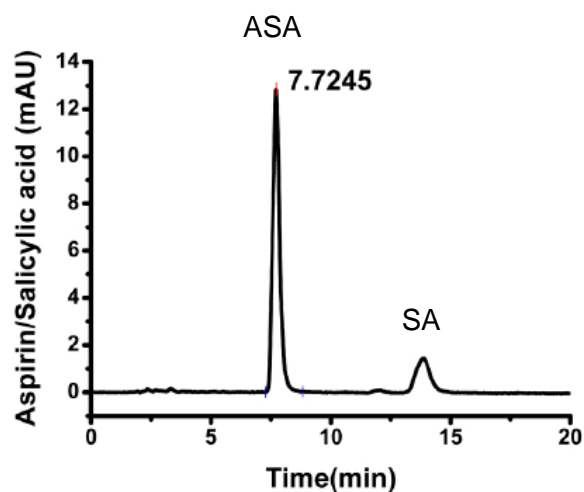


Figure 16. Heated human plasma does not hydrolyse aspirin. EDTA (1 mM) treated human plasma was heated at 75 °C for 2 hours. And then 100 µl heated plasma was incubated with 10 µg aspirin at 37°C for 2 hours. The reaction was stopped by adding 1% formic acid, aspirin and salicylic acid were extracted by ethyl acetate: diethyl ether (4:1, v/v). The organic phase recovered was dried under N₂. Aspirin and salicylic acid were then reconstituted in 500 µl of HPLC solvent (1% glacial acetic acid: methanol, 60:40, v/v) and 50 µl was analyzed by RP-HPLC.

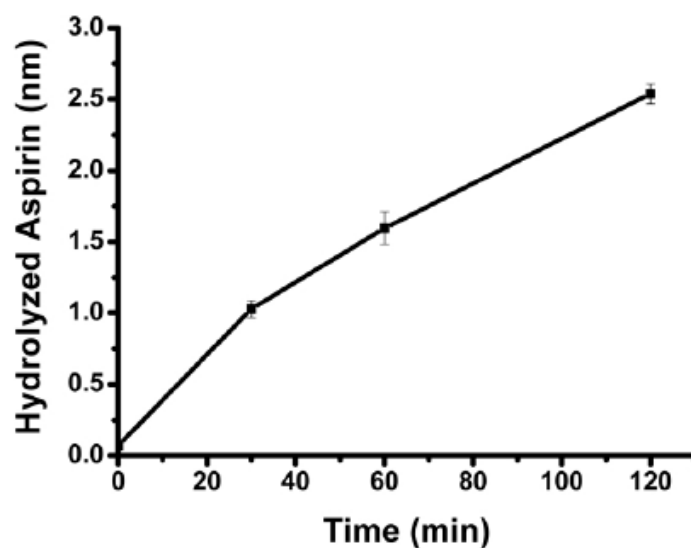


Figure 17. Time course of Aspirin hydrolysis. Aspirin (50 μg) in 500 μl PBS was incubated with 500 μl human plasma and at each time point (0, 30, 60, 120min) 200 μl mixture was removed before the reaction was stopped by adding 1% formic acid and aspirin and salicylic acid were extracted by ethyl acetate: diethyl ether (4:1, v/v). The organic phase was separated and dried under N_2 , aspirin and salicylic acid were reconstituted in 500 μl of HPLC solvent (1% glacial acetic acid: methanol, 60:40, v/v) and 50 μl was analyzed by RP-HPLC. Data are presented as Mean \pm S.D., $n > 3$.

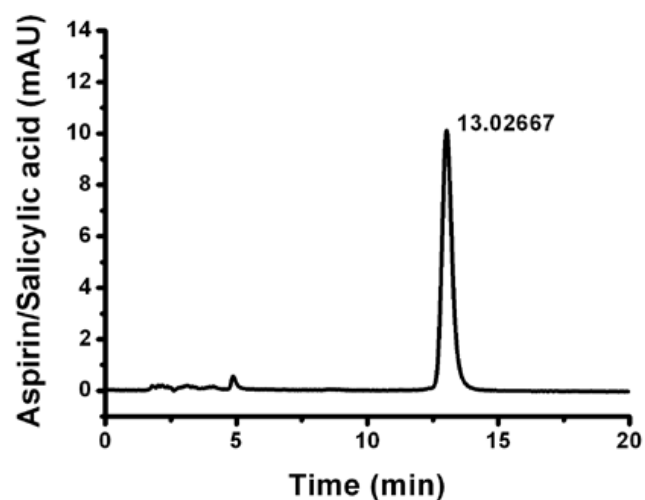


Figure 18. EDTA does not inhibit aspirin hydrolysis by human plasma. Human plasma was treated with 2mM EDTA, which totally inactivates PON-1 activity (data not shown). This plasma (100 μ l) was incubated with 10 μ g aspirin at 37°C for 2 hours before reaction was stopped by adding 1% formic acid. Aspirin and salicylic acid were extracted by ethyl acetate: diethyl ether (4:1, v/v). The organic phase was separated and dried down under N₂. Recovered aspirin and salicylic acid in 500 μ l of HPLC solvent (1% glacial acetic acid: methanol, 60:40, v/v) was analyzed by RP-HPLC.

3.2.2. PON1 is not an aspirin hydrolase

PON1 is a Ca^{2+} dependent enzyme and has been shown to hydrolyze aspirin (Santanam and Parthasarathy 2007). However, I found EDTA anti-agglutinated plasma had intact aspirin hydrolytic activity, suggesting PON1 lacks aspirin hydrolytic activity. PON1 is an HDL associated protein, as I first isolated HDL and LDL from acid citrate dextrose (ACD) anti-agglutinated plasma by KBr gradient density centrifugation and collected it into 18 fractions from bottom to top. Both aspirin hydrolytic activity and aryl esterase activity were examined in each fraction (Figure 19). Aspirin hydrolytic activity did not co-localize with aryl esterase activity. Aspirin hydrolytic activity was found in fraction 1 and 2 (soluble material), but aryl esterase activity or PON activity was found in fraction 4 to 6. Western blotting for PON1 and Apo A-I, an HDL protein, were performed to further confirm the location of PON1. I also examined recombinant PON1 for aspirin hydrolytic activity. Active recombinant PON1 did not hydrolyze aspirin at 2.5 μg , which had 2 times more aryl esterase activity than that of 10 μl plasma (Figure 20). Overall, PON1 cannot be an aspirin hydrolase, nor is any other lipoprotein-associated enzymes.

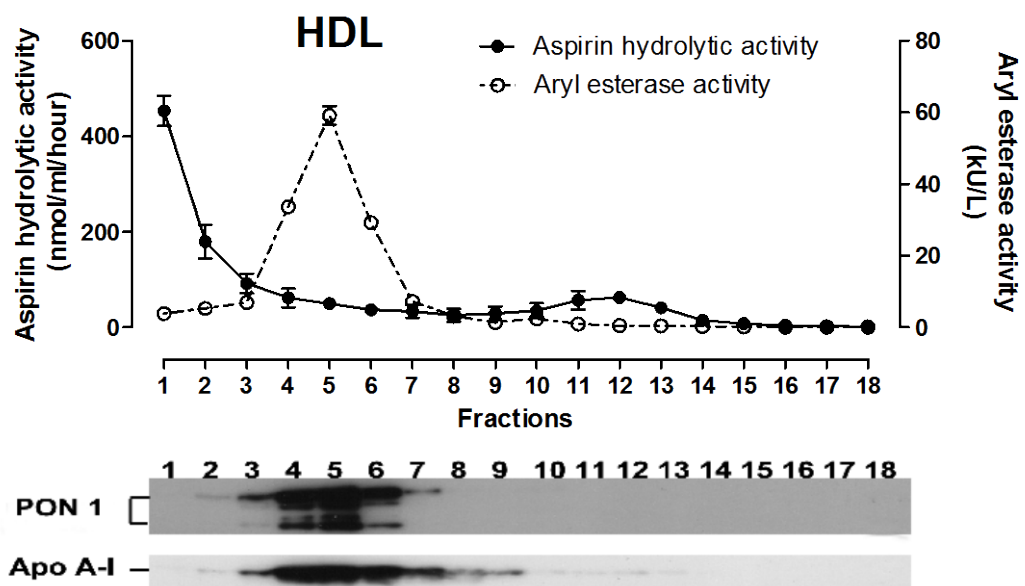


Figure 19. Aspirin hydrolytic activity does not co-localize with aryl esterase activity. Human plasma was subjected to density gradient (KBr) centrifugation. 20 fractions were collected (from HDL to VLDL). Each fraction is tested for aspirin hydrolysis and PA hydrolysis (aspirin hydrolytic activity and PON1 activity). A portion of each fraction was taken and was separated by SDS-gel electrophoresis. Western Immunoblotting was performed for PON-1 and Apo A-I.

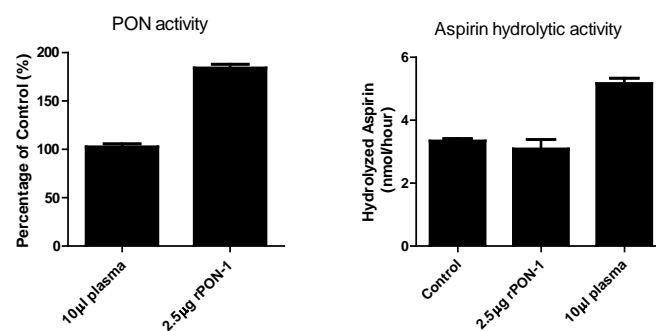


Figure 20. Recombinant PON1 does not hydrolyze aspirin. Recombinant PON1 does not hydrolyze aspirin. Arylesterase and aspirin hydrolytic activity of plasma and recombinant PON1 (2.5µg) were determined as above. Data are presented as mean \pm S.D., n=3.

3.2.3. Plasma platelet-activating factor acetylhydrolase shows weak aspirin hydrolytic activity.

Platelet-activating factor acetylhydrolase (PAFAH) hydrolyzes the acetyl group at the Sn-2 position of platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), which inactivated it. I examined if PAFAH accounted for plasma aspirin hydrolytic activity. PAFAH activity was measured in 18 plasma density gradient centrifugation fractions (Figure 21A). I found that 2/3 of the PAFAH activity was in LDL and 1/3 of the PAFAH activity was in HDL corresponding to the expected physical distribution, which did not collocate with aspirin hydrolytic activity (Figure 19). I calculated amount of plasma PAFAH in the lipoprotein fraction, which was about 0.05 μ g. I used the same amount of recombinant plasma PAFAH (0.05 μ g) to measure aspirin hydrolytic activity, but could not detect activity. However, 2 μ g recombinant plasma PAFAH did display detectable aspirin hydrolytic activity (Figure 21B). This suggests that aspirin is a poor substrate of plasma PAFAH.

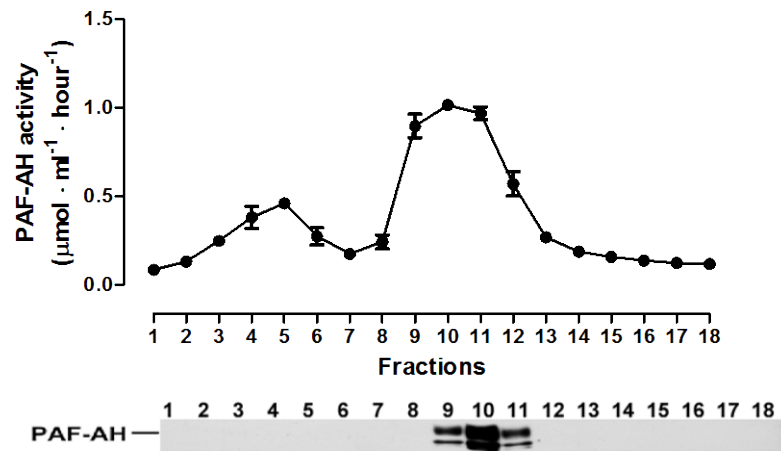
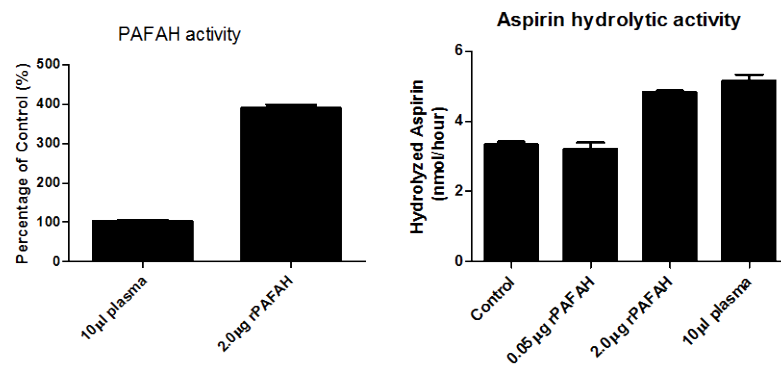
A**B**

Figure 21. Abundance of plasma Platelet activating factor – acetylhydrolase (PAF-AH) in LDL. Human plasma was subjected to density gradient (KBr) centrifugation as before. 20 fractions were collected (from HDL to VLDL). A portion of each fraction was taken and incubated with [^3H -acetyl]PAF at 37°C for 15 min. Hydrolyzed ^3H acetate was separated and collected on a C18 column. PAF-AH activity was calculated by converting the counts of [^3H]acetic acid produced by hydrolyzed [^3H -acetyl]PAF. (A). A portion of each fraction was separated by SDS-PAGE and western immunoblotting was performed for plasma PAF-AH (B). LDL fractions do not hydrolyze aspirin. Data are presented by mean \pm S.D., $n > 3$.

3.2.4. Butyrylcholinesterase cannot account for all of the plasma aspirin hydrolytic activity.

I examined Butyrylcholinesterase (BChE) for aspirin hydrolytic activity. Purified plasma BChE hydrolyzed aspirin efficiently, which was inhibited by eserine, a selective inhibitor of BChE (Figure 22). However, BChE and total plasma aspirin hydrolytic activity were not congruous with each other. I measured both aspirin hydrolytic activity and BChE activity which used butyrylthiocholine as a substrate (Figure 23). Ratios of plasma aspirin hydrolytic activity to BChE activity were not consistent in 14 donors. This suggests that BChE is not the only plasma aspirin hydrolase.

I purified plasma aspirin hydrolytic activity and examined amount of BChE in purified plasma aspirin hydrolase by comparing with BChE standard (Figure 24A). I found BChE only contributes in part to plasma aspirin hydrolysis. Fraction 1 (800 µg protein), where all the aspirinase activity of plasma existed, has an aspirinase activity more than 200ng BChE has. But western blotting only detected about 50 ng BChE in Fraction 1 (800 µg). For confirmation, I examined BChE null plasma, which totally lacks Butyrylthiocholine hydrolytic activity because of a silent mutation, for aspirin hydrolysis (Figure 24B). Surprisingly, I found normal plasma aspirin hydrolytic activity in the BChE null plasma. Albumin was therefore examined for aspirin hydrolytic activity as a potential catalyst since it deacelates aspirin (Liyasova, Schopfer et al. 2010). I examined location of albumin and ability of aspirin hydrolysis (Figure 25). The location of albumin in density gradient centrifugation fractions did

not localize with aspirin hydrolytic activity, although a tiny amount aspirin hydrolysis was detected in the albumin rich fractions (Burch and Blazer-Yost 1981).

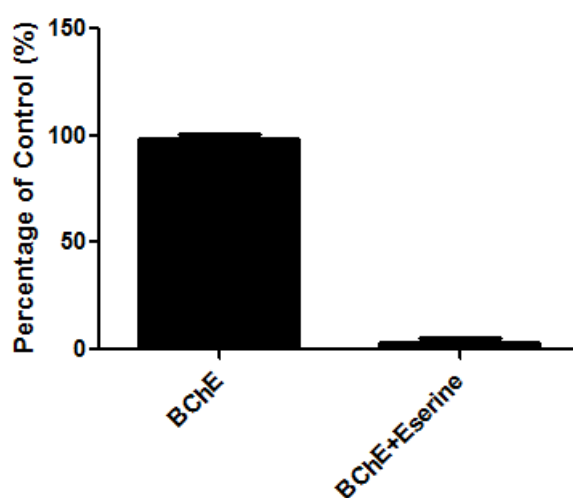


Figure 22. BChE hydrolyzes aspirin. 200 ng purified plasma BChE was incubated with 4 mM aspirin at 37°C for 2 hours in the presence or absence of eserine. Salicylic acid product was quantified by RP-HPLC. Data are present as mean \pm S.D., n=3.

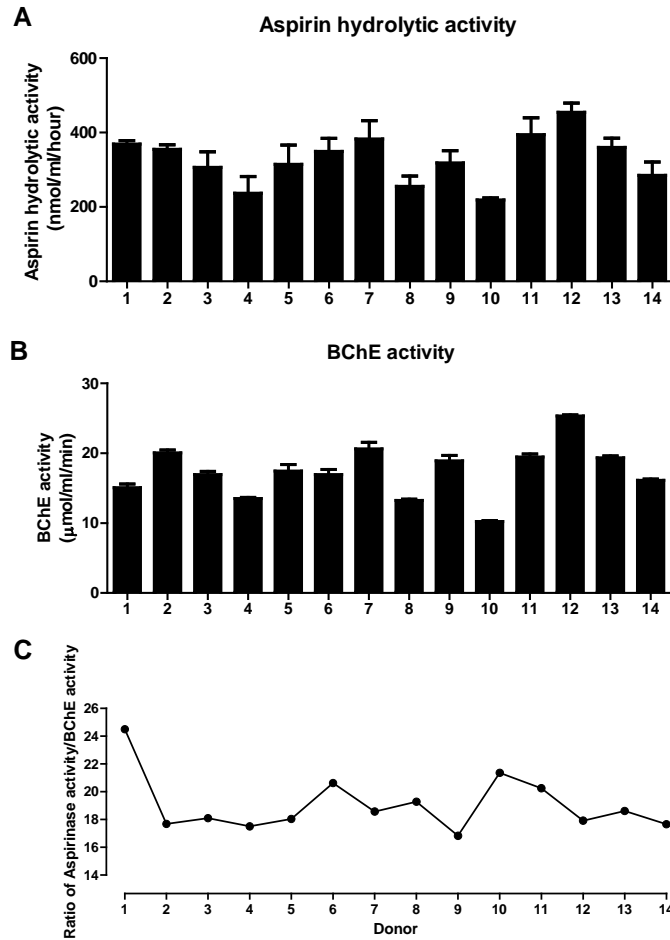


Figure 23. Aspirin hydrolytic activity and BChE activity in plasma of 14 donors. (A) Aspirin hydrolytic activity was measured in 14 donors' plasma. (B) BChE activity was measured in 14 donors' plasma. (C) The ratio of the two activities was calculated by mean (C). Data are presented as mean \pm S.D., n=3.

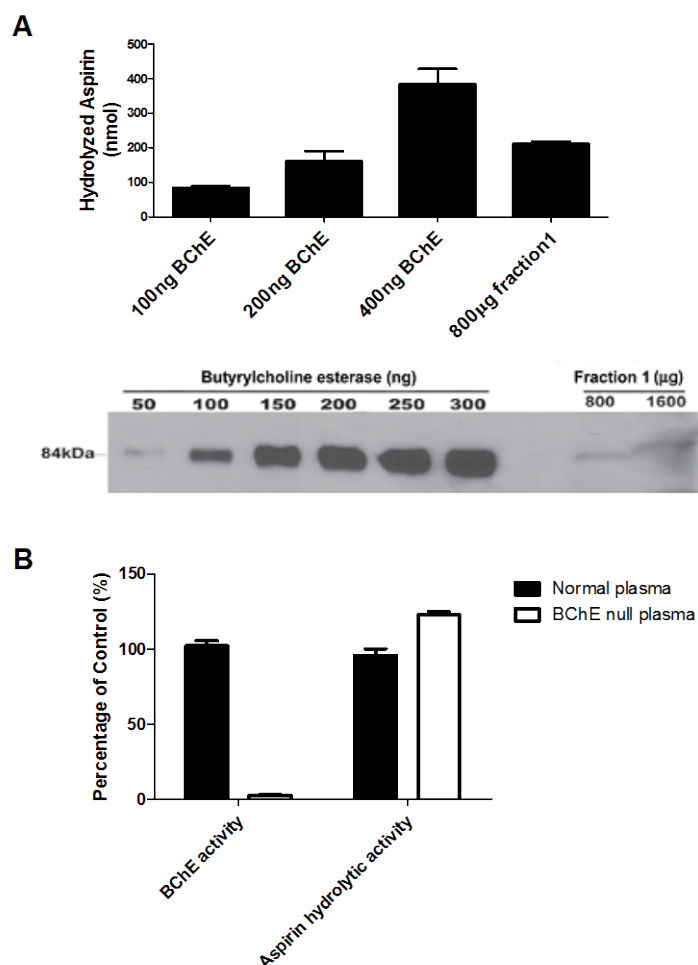


Figure 24. Butyrylcholinesterase appears to contribute little to plasma aspirin hydrolysis. (A) Plasma butyrylcholinesterase is a minor contributor to aspirin hydrolysis. Plasma was separated by density gradient centrifugation and fraction 1 containing aspirin hydrolytic activity was partially purified by Cibacron® blue chromatography. Butyrylcholinesterase protein in this fraction was assessed by western blotting (*top*) or aspirin hydrolytic activity (*bottom*) quantified by HPLC in comparison to known quantities of butyrylcholinesterase purified from human plasma. (B) Butyrylcholinesterase and aspirin hydrolytic activity were determined in normal human plasma and plasma from an individual with an inactivating null mutation in butyrylcholinesterase. Data are presented as mean \pm S.D., n=3.

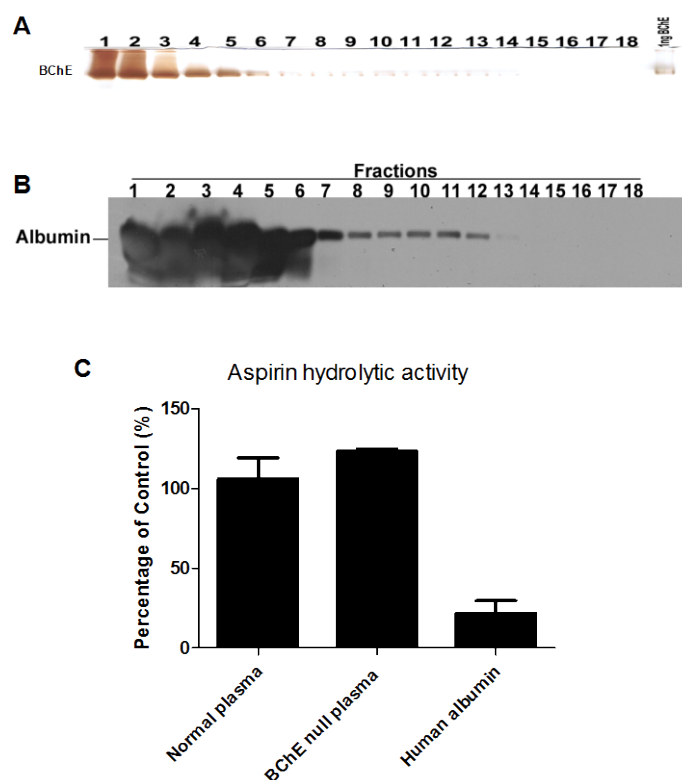


Figure 25. Nondenaturing gel stained for BChE activity with butyrylthiocholine. (A) 10 μ l of each fraction (KBr gradient) is separated by a 10% native polyacrylamide gel. Fraction 1, 2 and 3 were shown BChE tetramers, trimers, dimers, and monomers. Staining procedure is described in Method part. (B) Albumin western blotting of each fraction. (C) Aspirin hydrolysis in plasma and albumin. Normal, BChE null plasma, and albumin (adjusted to physiological concentration, 0.5%) (10 μ l) are incubated with 4 mM aspirin at 37°C for 2 hours, and then quantitate aspirin hydrolysis. Data are present as mean \pm S.D., n=3.

3.2.5. Composition of plasma aspirin hydrolytic activity

I measured both butyrylthiocholine hydrolytic activity (BTC activity) and aspirin hydrolytic activity of BChE and BChE null plasma to assess non-BChE plasma aspirin hydrolase activity. The ratio of BTC activity to aspirin hydrolytic activity shows the two activities are distinctive (Table 1). For BChE, the ratio is 21.81; for BChE null plasma, the ratio is 0.35. These data can be used to estimate the relative contribution of the two aspirinase in plasma to total aspirin hydrolysis, using these ratios to define overlap in activities. If I set the percentage of plasma aspirin hydrolytic activity coursed by BChE as x and that by non-BChE aspirin hydrolase as y , I obtain Equation 1, where z stands for real ratio of BTC activity to aspirin hydrolytic activity in plasma. I measured the ratio of BTC activity to aspirin hydrolytic activity in 14 donor's plasma and calculated the percentage composition of plasma aspirin hydrolytic activity (Figure 26). This shows that the theoretical compositions of BChE and non-BChE aspirin hydrolases are about equaul.

Table 1

	BChE	BChE null plasma
BTC activity (U/ml/min)	78.07	1.49
Aspirin hydrolytic activity (nmol/ml/min)	3.58	4.28
Ratio (BTC/Aspirin hydrolytic activity)	21.81	0.35

n=3; data presented by Mean; 1U=265.2nmoles

Equation 1

$$x + y = 1$$

$$21.81x + 0.35y = z$$

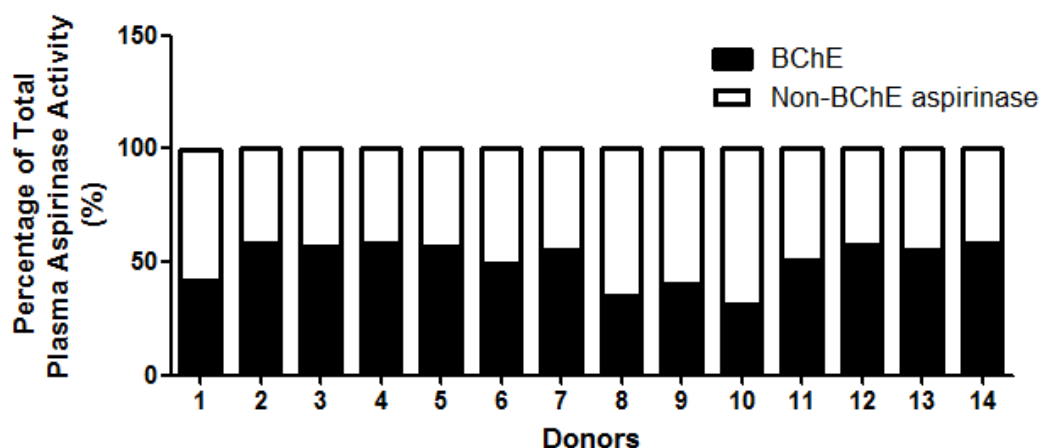


Figure 26. Theoretical composition of plasma aspirin hydrolytic activity.

According to the formula, composition of plasma aspirin hydrolytic activity was calculated.

3.2.6. Butyrylcholinesterase accounts for variation of plasma aspirin hydrolytic activity

The above approach calculated the composition of plasma aspirin hydrolytic activity and suggested the conclusion that two enzymes at least in plasma hydrolyze aspirin. I found significant variation among individuals and then used inhibitors to show the contribution of the two enzymes in those donors. First I investigated plasma aspirin hydrolytic activity in 7 donors, and found significant variation (Figure 27A). The maximum of plasma aspirin hydrolytic activity was about 1482 nmol/ml/hour and the minimum of plasma aspirin hydrolytic activity was about 220 nmol/ml/hour with a mean of about 500 nmol/ml/hour. Oxidized ATP was found to inhibit aspirin hydrolytic activity in BChE null plasma, and inhibited little that of pure BChE, so it can be used to assess non-BChE activity. Conversely, procainamide inhibited aspirin

hydrolytic activity of BChE, and accordingly had little effect in null plasma (Figure 27B). Oxidized ATP effectively inhibited recombinant PAFAH1b2. I used these two inhibitors to elucidate the actual composition of plasma aspirin hydrolytic activity (Figure 27C). I found BChE accounts for the variation of plasma aspirin hydrolytic activity, and that non-BChE aspirin hydrolase activity was fairly constant. Tris buffer, an activity enhancer of BChE, significantly increases aspirin hydrolysis by plasma, but did change the variation trends in the 7 donors (Figure 28). These suggest that BChE contributes the variation of aspirin hydrolytic activity in the donors.

I then examined aspirin hydrolytic activity in 2,275 serum samples to determine if the variation was genetically encoded and if so, if both BChE and non-BChE enzyme were responsible. Again I found significant variation in this large population. The highest activity was about 37 nmol/ml/min and the lowest activity was about 3 nmol/ml/min. The difference was up to 12-fold (Figure 29A). Genome-wide association analysis was performed to map serum aspirin hydrolytic activity to the entire genome. BChE is the only gene related to serum aspirin hydrolytic activity variation (Figure 29B). The p value is 6.4×10^{-14} . All together, BChE contributes variation of plasma aspirin hydrolytic activity and this is genetically encoded.

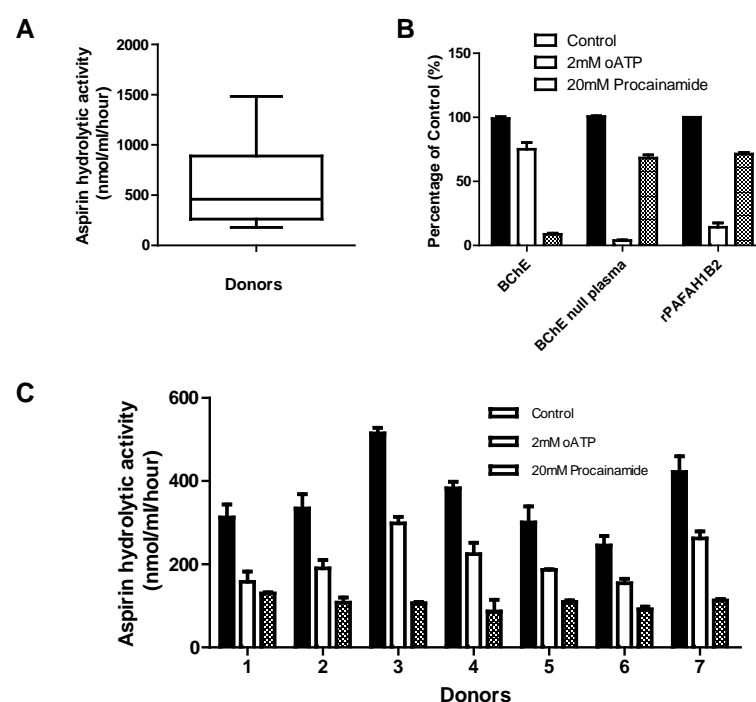


Figure 27. Aspirin hydrolysis in plasma is variable, with variable contribution by butyrylcholinesterase. (A) Box and whisker plot of plasma aspirin hydrolytic activity. Aspirin hydrolysis by plasma of seven donors is depicted with maximum (1482 nmol/ml/h), minimum (220 nmol/ml/h), lowest and highest quartile and the median. (B) Both butyrylcholinesterase and at least one other activity contribute to aspirin hydrolysis in normal human plasma. Aspirin hydrolytic activity in normal plasma, BChE null plasma, and recombinant PAFAH1B2 were examined in the presence of procainamide to inhibit butyrylcholinesterase and oxidized ATP to inhibit non-butyrylcholinesterase activity. (C) Plasma aspirin hydrolytic activities vary among donors, but largely from difference among BChE activity. Aspirin hydrolysis in plasma from distinct donors in the presence of procainamide or oxidized ATP was quantified as above. Data are presented as mean \pm S.D., n=3.

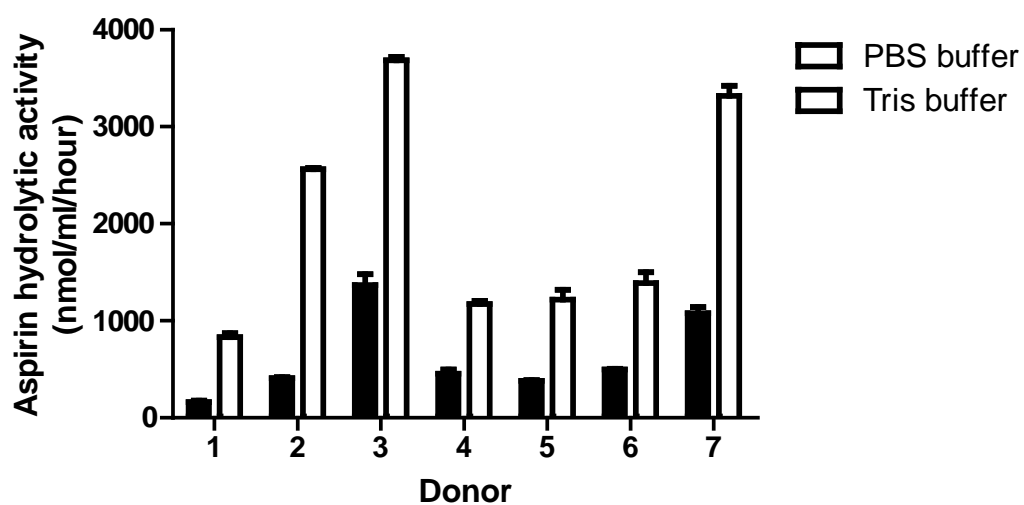


Figure 28. Tris buffer enhances aspirin hydrolysis by plasma. 4 mM aspirin was incubated with 10 μ l plasma in PBS buffer or Tris buffer at pH 7.4 at 37°C for 2 hours. Aspirin hydrolyzed was quantified by RP-HPLC.

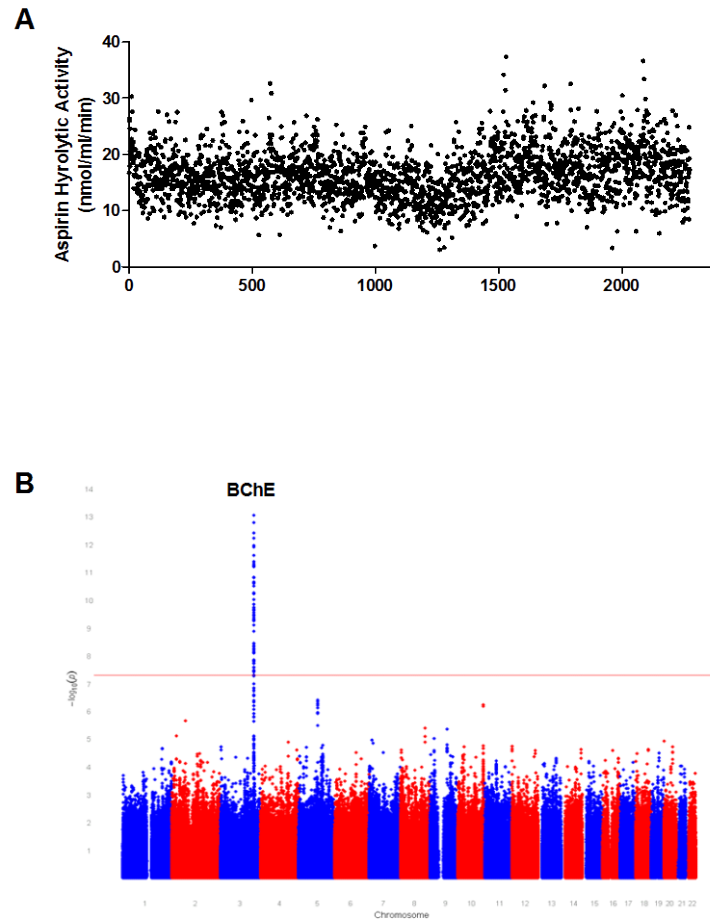


Figure 29. Variation in serum aspirin hydrolytic activity is widespread, and genetic variation primarily correlates to butyrylcholinesterase. (A) High throughput HPLC analysis of aspirin hydrolysis in 2,275 serum samples. Analysis used a single measurement, which was validated by randomly re-analysis of 285 samples. (B) Manhattan plot of genome-wide p values for association with serum aspirin hydrolytic activity.

3.2.7. Type I PAFAH accounts for non-BChE plasma aspirin hydrolytic activity.

I identified type I PAFAH as erythrocyte aspirin hydrolase (Chapter IV). When I identified bands responsible for erythrocyte aspirin hydrolytic activity, one of the candidates is ATPase. So ATP was used as an inhibitor and aspirin hydrolytic activity of type I PAFAH was inhibited by ATP (Figure 30). I used oxidized ATP as an inhibitor for erythrocyte aspirin hydrolase, which is more stable than ATP. It also inhibited type I PAFAH as ATP. I also examined effects of oxidized ATP and procainamide on aspirin hydrolytic activity of type I PAFAH. The same as non-BChE plasma aspirin hydrolase, type I PAFAH also inhibited by oxidized ATP, but not inhibited by procainamide (Figure 31). This made me think the possibility that type I PAFAH may account for non-BChE plasma aspirin hydrolytic activity. I did western blotting for PAFAH1B2 in density gradient centrifugation fractions, which I knew aspirin hydrolytic activity was in fraction 1. PAFAH1B2 was detected in fraction 1 where most aspirin hydrolytic activity existed (Figure 32). To confirm, western blotting for PAFAH1B2 was also performed in BChE null plasma along with 7 other plasmas. PAFAH1B2 was found in all plasmas, especially in BChE null plasma. Another phenomenon was that serum had more aspirin hydrolytic activity than plasma from same donor (Figure 33). These suggest aspirin hydrolytic activity of BChE null plasma is from type I PAFAH and type I PAFAH accounts for parts of plasma aspirin hydrolytic activity.

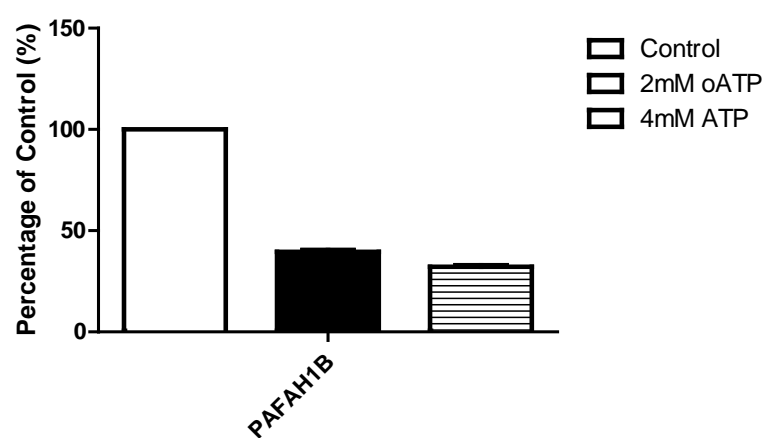


Figure 30. PAFAH1B is inhibited by ATP and oxidized ATP. 4 mM aspirin was incubated with purified type I PAFAH at 37°C for 2 hours in the presence or absence of ATP or oxidized ATP and followed by measurement of aspirin hydrolytic activity. Data are normalized to control and presented by mean \pm S.D., $n \geq 3$.

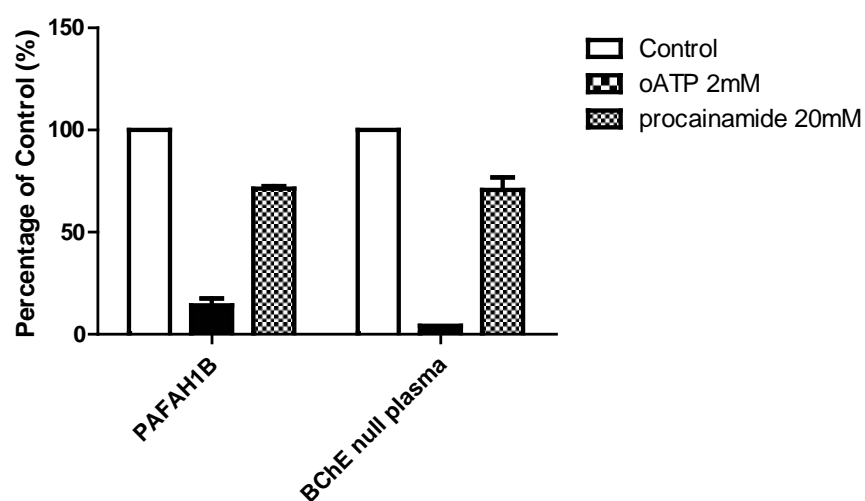


Figure 31. Both PAFAH1B and BChE null plasma were inhibited by oxidized ATP, but little inhibited by procainamide. Purified erythrocyte aspirin hydrolase was incubated with 4 mM aspirin in the presence or absence of inhibitors (2 mM oxidized ATP and 20 mM procainamide) at 37°C for 2 hours. Hydrolyzed aspirin was measured by RP-HPLC. Data are presented by mean \pm S.D., $n \geq 3$.

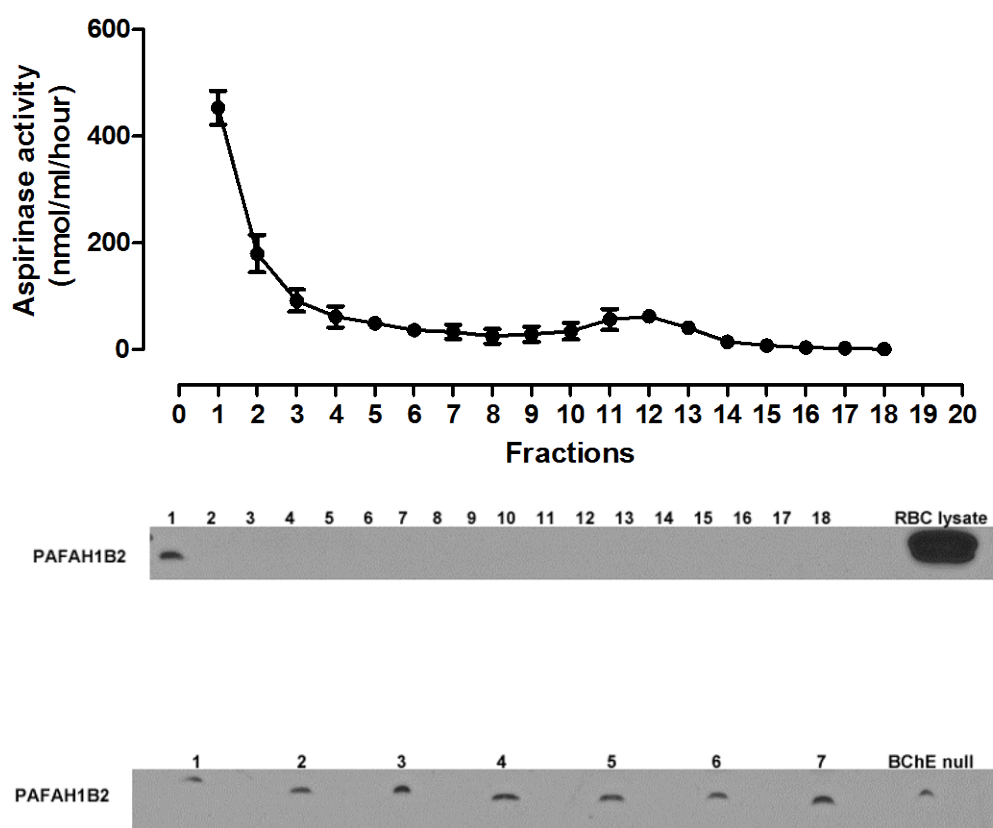


Figure 32. Type 1 PAFAH accounts for aspirin hydrolytic activity of BChE null plasma. Aspirin hydrolytic activity was measured in plasma density gradient centrifugation fractions. Each fraction was immunoblotted by PAFAH1B2 with erythrocyte lysate as a positive control. Only fraction 1 was detected PAFAH1B2. Immunoblot for PAFAH1B2 was performed in BChE null plasma along with seven different normal plasmas. PAFAH1B2 was detected in all plasmas. Data are presented by mean \pm S.D., $n \geq 3$.

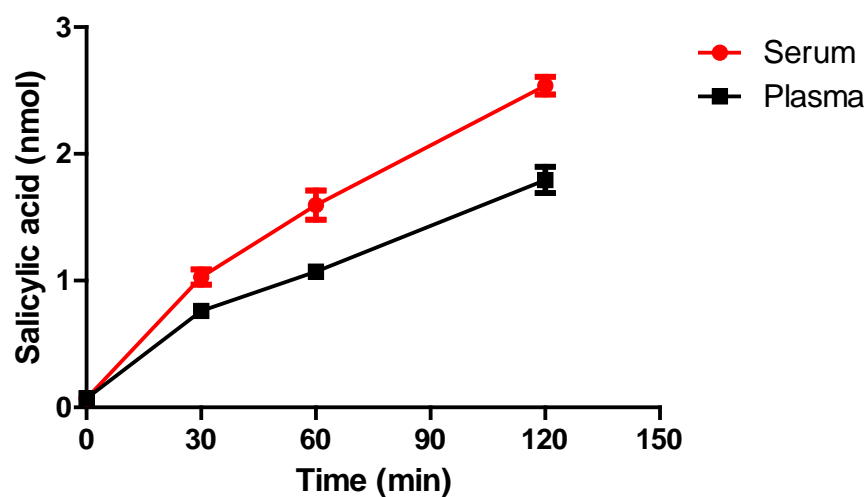


Figure 33. Comparison of time course aspirin hydrolysis by serum and plasma. 4 mM aspirin was incubated with 1ml of serum or plasma at 37°C. 50 μ l was taken out at each time point and followed quantification of salicylic acid by RP-HPLC. Data are presented by mean \pm S.D., $n \geq 3$.

3.3. Discussion

Aspirin is used worldwide medically to prevent cardiovascular and thrombotic diseases. However, not all individuals or members of groups appear to receive the benefits of aspirin administration. In recent decades more cases of aspirin treatment failure drew people's attention, which was commonly termed "aspirin resistance", but whether the phenomenon of "aspirin resistance" is a valid description, or even identifiable given difficulties in clinical measurement of platelet function (Santilli, Rocca et al. 2009), is debated (Fitzgerald and Maree 2007; Patrono and Rocca 2007). Clinical studies showed that aspirin resistance in population is about 8-45% (Grottemeyer 1991; Grottemeyer, Scharafinski et al. 1993; Helgason, Tortorice et al. 1993; Gum, Kottke-Marchant et al. 2001). The wide range of percentage of aspirin resistance observed is because of methods used to identify it. Resistance to aspirin can be identified in *ex vivo* assays of platelet reactivity, in *in vivo* studies (Fitzgerald and Maree 2007), or the apparent lack of efficacy in defined populations (Fitzgerald and Maree 2007; Patrono and Rocca 2007; Lev 2009; Santilli, Rocca et al. 2009; Pignone and Williams 2010). A molecular basis for insensitivity to aspirin could include events that depend on thromboxane A₂ signaling pathways, but also would include aspirin bioavailability as observed in populations associated with aspirin insensitivity (Williams, Asad et al. 1987; Akopov, Grigorian et al. 1992; Puche, Gomez-Valverde et al. 1993; Gresner, Dolnik et al. 2006).

Although aspirin has been in massive use since its introduction in the 1890s, its metabolism in blood remains undefined. Aspirin is not a physiologic metabolite

whose metabolism was subject to selection, so its hydrolysis reflects activity of esterases that can accept it as a substrate. However, the key enzymes responsible for the process of metabolism are still unknown. Aspirin acetylates albumin (Yang, Bian et al. 2007) that reversibly binds and transports the drug (Costello and Green 1987), and circulating esterases paraoxonase-1 (Santanam and Parthasarathy 2007) and butyrylcholinesterase (Masson, Froment et al. 1998) are able to accept aspirin as a substrate and hydrolyze this simple salicylate ester.

I detected about 20% of aspirin hydrolytic activity in purified human serum albumin comparing with normal plasma containing same amount of albumin, but it still cannot account for the aspirin hydrolytic activity in BChE null plasma. Location of albumin in gradient density centrifugation fractions (1-6) was distinct from the location of aspirin hydrolytic activity (fraction 1) which co-located with BChE. Albumin was reported having esterase activity (Morikawa, Inoue et al. 1979; Rainsford, Ford et al. 1980), but this was thought as “psuedoesterase activity” due to non-catalytic acetylation (Lockridge, Xue et al. 2008). So albumin actually cannot be an enzyme which responsible for plasma aspirin hydrolytic activity.

Butyrylcholinesterase circulates as a tetramer that displays complex kinetics (Simeon-Rudolf, Reiner et al. 1999) subject to both environmental and genetic variation that obfuscates catalysis (Goodall 2004). Plasma aspirin hydrolytic activity is not normally distributed (Adebayo, Williams et al. 2007) and genetic variation affects butyrylcholinesterase activity that correlates with distinct neuromuscular

blockade in response to the anesthetic succinylcholine (Levano, Ginz et al. 2005), a butyrylcholinesterase substrate. Variation in plasma aspirin esterase activity additionally correlates with measures of type 2 diabetes (Gresner, Dolnik et al. 2006; Kotani, Kimura et al. 2010) that may be relevant to aspirin sensitivity.

I sought to identify the activity (ies) in plasma that hydrolyzed aspirin, rather than testing candidate enzymes for aspirin hydrolytic activity, by quantifying the salicylic acid product, unlike the acetyl group, that is not sequestered into non-enzymatic adducts. PON-1 was shown having aspirin hydrolytic activity (Santanam and Parthasarathy 2007), but my data did not support it. Density gradient separation shows the plasma aspirin hydrolytic activity is soluble, and therefore cannot result from the lipoprotein associated esterase paraoxonase-1 even though this activity is suggested as plasma aspirin hydrolase (Santanam and Parthasarathy 2007). As we know PON-1 binds to Cibacron blue, but Cibacron blue binding proteins were not detected any aspirin hydrolytic activity (not shown). Finally, functionally recombinant PON-1 failed to hydrolyze aspirin. However, aspirin use was reported associated with higher serum concentration of PON-1 (Blatter-Garin, Kalix et al. 2003), which was believed because aspirin induced PON-1 expression (Jaichander, Selvarajan et al. 2008).

I sought to define the role of circulating butyrylcholinesterase on aspirin metabolism by assessing aspirin hydrolysis in plasma obtained from an individual with a functional knockout as a consequence of a single, defined mutation (Nogueira,

McGuire et al. 1990). BChE null plasma still can hydrolyze aspirin efficiently, strongly indicating that BChE was not the only aspirin hydrolytic enzyme accounting for plasma or serum aspirin hydrolytic activity. Results from this classic approach would, and initially did, exclude this enzyme from consideration. Plasma deficient in this single enzyme, however, did reduce the complexity of aspirin hydrolysis, and complete inhibition of aspirin hydrolytic activity in this BChE deficient individual's plasma by oxidized ATP was consistent with the presence of a single hydrolytic enzyme. Inhibition by oxidized ATP showed aspirin hydrolysis by this activity varied, up to two fold, among donors. Conversely, the butyrylcholinesterase inhibitor procainamide showed it too inhibited plasma aspirin hydrolytic activity, and that the effectiveness of this inhibition also varied among donors. Together the data suggests butyrylcholinesterase does participate in aspirin hydrolysis in plasma along with a currently unidentified esterase. The two plasma aspirin hydrolytic activities vary independently. Six-fold difference of aspirinase activity was observed coursing by BChE among donors, but little variation was observed in the unidentified esterase. With the identity uninformed of the non-BChE plasma aspirin hydrolase (BChE null plasma), I was still able to characterize the composition of them in plasma by their different substrates. BChE can hydrolyze both butyrylcholine and aspirin, but non-BChE aspirin hydrolase can only hydrolyze aspirin. I measured both two activities (aspirin and butyrylcholine) and calculated the ratio of the two (Table 1). By using the two ratios, I proposed a formula and calculated theoretically percentage composition

of the two portions of activities. Results suggest that it is about half to half, which is consistent with the results of inhibition studies.

I determined if a component to the variation of aspirin hydrolysis was genetic through a genome-wide association study (GWAS). When I expanded the number of individual plasmas examined to 2,275, I discovered strong variation, up to 12-fold, in plasma aspirin hydrolysis. Adebayo GI *et al.* found 7-fold difference of serum aspirin hydrolytic activity in 107 individuals (Adebayo, Williams et al. 2007). Smaller samples studied may be the reason of lower difference observed. The activity range Adebayo GI *et al.* reported was 33.90 nmol/ml/min to 222.65 nmol/ml/min, which is higher than what I found (3.062 nmol/ml/min to 37.38 nmol/ml/min). The reason of the difference may be because of different assay buffer used. 50 mM Tris (pH 7.2) buffer can increase plasma aspirin hydrolytic activity up to 6 fold comparing with that of PBS buffer at the same pH used. It has been shown as a character of normal BChE but not atypical BChE (La Du 1971). I also determined that this strongly associated with a single SNP located at the end of chromosome 3, so a single locus accounted for all of the significant variation in activity. Plasma aspirin hydrolysis is therefore genetically determined, and the SNP shows this locus is that encoding butyrylcholinesterase. GWAS has therefore identified this gene where biochemical and knockout strategies were ineffective.

Characterization of non-BChE aspirin hydrolase by inhibitors reveals that the undetermined plasma aspirin hydrolase can be inhibited by oxidized ATP, but not by

procainamide, a selected inhibitor of BChE. I found recombinant PAFAH1b2 was inhibited by oxidized ATP and it was detected by Western Blot in BChE null plasma and normal plasma as well. These suggest that PAFAH1B accounts for the non-BChE plasma aspirin hydrolytic activity, which contributes about half of plasma aspirin hydrolytic activity. By inhibition studies, this activity has low variation among donors, which may be the reason that GWAS failed to detect it.

My findings reveal that significant variation of serum aspirin hydrolytic activity exists among people, resulting from genetic BChE variation, in addition to varied amount of two aspirin hydrolases. The non-BChE aspirin hydrolytic activity is because of type I PAF acetylhydrolase. This may provide molecular basis for explanations of aspirin “resistance” and a starting point for personalized medicine of aspirin.

CHAPTER IV

4. IDENTIFICATION AND CHARACTERIZATION OF ASPIRIN HYDROLASE IN HUMAN ERYTHROCYTES

4.1. Introduction

Since the anti-platelet function of aspirin primarily because of its acetyl group, it is important to know the pathways of losing acetyl group in blood. Aspirin acetylates cyclooxygenase-1 to suppress platelet activation, which most likely is controlled by rate of its hydrolysis. After first pass metabolism, about 70% of aspirin reaches blood and rapidly is hydrolyzed to salicylate enzymatically (Seymour, Williams et al. 1984). Therefore aspirin has a very short half-life (15-20min) in human circulation (Levy 1965; Rowland, Riegelman et al. 1972). Enzymes catalyzing aspirin hydrolysis play important roles in controlling lifespan of aspirin in blood and further negatively affecting its anti-platelet function. Plasma butyrylcholinesterase is a major aspirin hydrolytic activity (Rainsford, Ford et al. 1980) in blood. I have shown that a significant variation of plasma aspirin hydrolytic activity existed in population, which is up to 12-fold. And I also showed that the variation is associated with BChE genetically, although BChE is not the only plasma aspirin hydrolase.

As reported, aspirin hydrolases of erythrocytes (Reilly and FitzGerald 1988) and plasma (O'Brien 1968) hydrolyze aspirin in the systemic circulation in a first order process. Enzymes able to hydrolyze aspirin are present in blood and have been termed "aspirin esterases". Most publications in this field seem to ignore the contribution of the red cell to aspirin hydrolysis. Several previous reports have mentioned in passing that whole blood has a higher enzymatic activity than serum or plasma, but the contribution from the cellular fraction has not been extensively studied (Harthon and Hedstrom 1971). The first time Rylance & Wallace showed that there was no significant correlation between the enzyme levels in erythrocytes and plasma suggesting that the enzyme inside the red cell and plasma was different (Rylance and Wallace 1981). Human erythrocytes are anucleate and without mitochondria in mammals. They live out a finite life span (120 days) and are unable to renew any of its critical enzymes and structural proteins, although it is able to repair some oxidative damage to its component parts. Erythrocytes quickly complete one cycle of circulation in an average of 20 seconds (Pierige, Serafini et al. 2008), which makes available to aspirin immediately after it is absorbed. In addition, aspirin permeates rapidly into the erythrocytes, almost within one minute (Ohsako, Matsumoto et al. 1993). Thus, erythrocytes are important in controlling aspirin hydrolysis in normal whole human blood. Erythrocyte aspirin hydrolase was first concluded as a membrane-bound acetylcholinesterase by Costello, P.B. et al. (Costello and Green 1982). But later they found it is located in cytosol, which is not affected by Ca^{2+} and other bivalent cations and is not acetylcholine esterase (Costello

and Green 1983). A 95,000 Dalton protein was partially purified as a major aspirin hydrolytic hydrolase in human erythrocytes. But the identity of the erythrocyte aspirin hydrolase is still unclear. They also found hematocrit positively correlated with the rate of aspirin hydrolysis (Costello, Caruana et al. 1984). An erythrocyte hydrolase was found to be defective in systemic lupus erythematosus (SLE), which resulted in reduced aspirin hydrolyzing capacity (Costello and Green 1986). *In vitro* studies reveal that about half of aspirin hydrolytic activity in blood is because of erythrocytes, which also consist with the phenomenon that whole blood hydrolyzing aspirin faster than that of plasma about twofold (Costello, Caruana et al. 1984).

A unique class of phospholipases A₂ has a property for deacylation of phospholipids Sn-2 acetyl residue of platelet-activating factor (PAF), called platelet-activating factor acetylhydrolase (PAFAH) (McIntyre, Prescott et al. 2009). Type I PAFAH, a unique serine esterase, is very specific for Sn-2 acetyl group hydrolysis and has significant homology with a diverse family of microbial hydrolases which have a series of bacterial polysaccharide as substrates but not with any other mammalian proteins (Arai, Koizumi et al. 2002). Its tertiary structure resembles that of G-proteins (Ho, Swenson et al. 1997). Type I PAFAH is expressed in diverse human tissue and has undefined biological functions, although anti-apoptotic action was showed (Bonin, Ryan et al. 2004).

In normal whole human blood, erythrocytes are the major place where aspirin is hydrolyzed. So it is important to identify and to characterize the aspirin hydrolases

from human erythrocytes. It will reveal the role of erythrocytes in modulation of anti-platelet function of aspirin.

4.2. Results

4.2.1. Erythrocytes account for half or more of blood aspirin hydrolytic activity, which varies inter-individually.

Erythrocytes (Costello and Green 1982) and plasma (O'Brien 1968) were both believed important in controlling aspirin hydrolysis in human circulation. I compared contribution of erythrocytes and plasma to whole blood aspirin hydrolytic activity in 13 donors (Figure 34). Contributions of whole blood aspirin hydrolytic activity by plasma and erythrocytes varied from donor to donor. I also did time course comparison of aspirin hydrolytic activity of erythrocytes and plasma from a single donor (Figure 35). Erythrocytes accounted for most of whole blood aspirin hydrolytic activity comparing to plasma in this single donor. I also investigated erythrocyte aspirin hydrolytic activity among eleven donors (Figure 36). Washed erythrocytes (10^8) from different donors were analyzed for aspirin hydrolytic activity. Over a 2-fold difference was found among donor cells. It suggests that erythrocytes are important in inactivating circulating aspirin, but the donor cells, too, vary on this attribute.

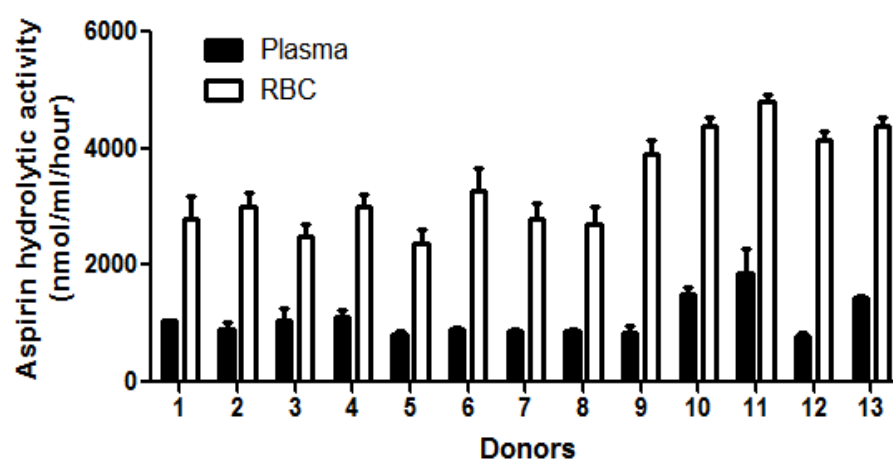


Figure 34. Comparisons of Aspirin hydrolytic activity of plasma and erythrocytes among 13 individuals. Plasma and erythrocytes were separated from same donor's whole blood. Same volume (10 μ l) of plasma and erythrocytes were measured for aspirinase activity as described in Chapter II. Data are present as mean \pm S.D., n=3.

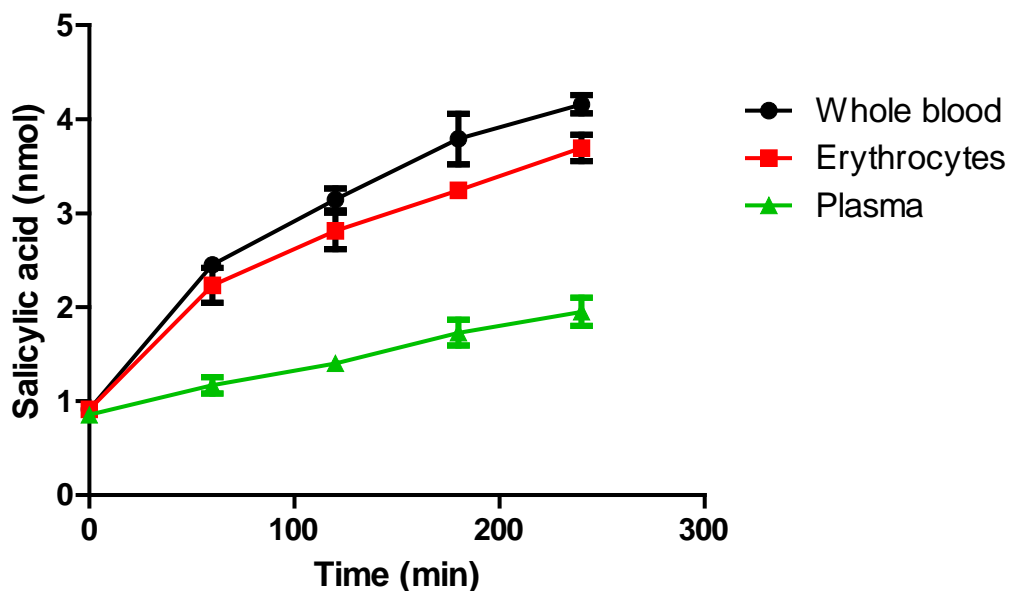


Figure 35. Time course hydrolysis of aspirin by whole blood, erythrocytes and plasma. Erythrocytes were isolated from ACD anti-aggregated blood by centrifugation at $1,000\times g$ for 30min at $4^{\circ}C$. White blood cells layer was carefully removed after plasma was collected. Volume ratio of erythrocytes to plasma was about 1 to 1. 4mM aspirin was incubated with whole blood, erythrocytes, or plasma in 500 μ l at $37^{\circ}C$. 50 μ l (whole blood) or 25 μ l (erythrocytes and plasma) was taken out from incubation at each time point to stop the reaction, and enzymatically produced salicylic acid was quantified by RP-HPLC. Data are presented by mean \pm S.D., $n\geq 3$.

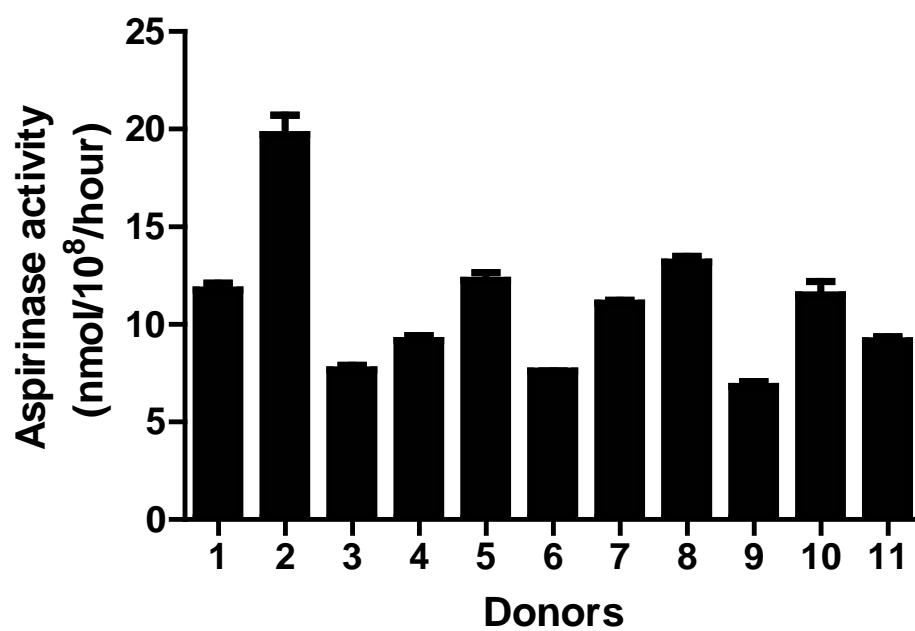


Figure 36. Aspirin hydrolytic activity variation of erythrocytes. Washed erythrocytes (10^8) of different donors were measured for aspirin hydrolytic activity as described in Chapter II. Data are present as mean \pm S.D., $n=3$.

4.2.2. Neither AChE nor BChE accounts for erythrocyte aspirin hydrolytic activity.

Both AChE and BChE were examined and neither of them was found had relationship with erythrocyte aspirin hydrolytic activity (Figure 37). BChE is a plasma aspirin hydrolase, which synthesized by liver. If there is any BChE in erythrocytes is not well documented, but it could contaminate with cells. Although AChE was showed not related with aspirin hydrolytic activity of erythrocytes, I still confirmed the conclusion before I started erythrocyte aspirin hydrolase purification. I directly examined purified AChE from human erythrocytes for aspirin hydrolytic activity (Figure 37A). No aspirin hydrolytic activity was detected in the active AChE which confirmed by α -naphthyl acetate staining. I also determined BChE amount by comparing with BChE standard (Figure 37B), which cannot account for aspirin hydrolytic activity in erythrocytes.

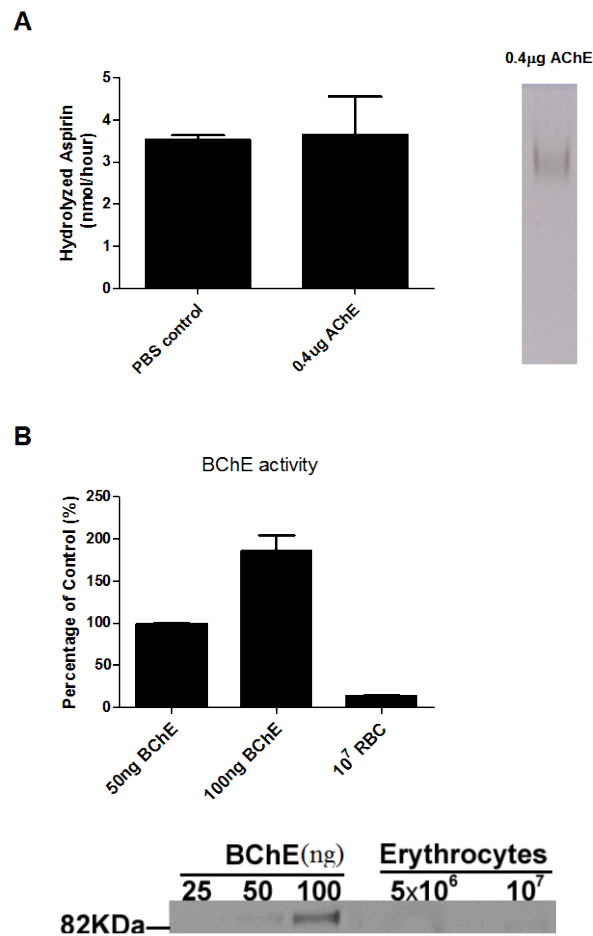


Figure 37. Neither AChE nor BChE accounts for erythrocyte aspirin hydrolytic activity. (A) 0.4 µg of active purified AChE from erythrocytes was incubated with 4 mM aspirin at 37°C for 2 hours. Hydrolyzed aspirin in one hour was calculated by quantifying produced salicylic acid from aspirin by a HPLC based method. (B) BChE was non-detectable in 10⁷ erythrocytes. I measured BChE activity by using butyrylthiocholine as a substrate and did western blotting for BChE in 10⁷ erythrocytes lysates. Data are present as mean ± S.D., n≥3.

4.2.3. Type I PAFAH is aspirin hydrolase of erythrocytes.

I purified erythrocyte aspirin hydrolytic activity by a combination of two DEAE columns, HiTrap Q column, and a sephacryl s-200 column (Figure 38). The overall purification was 1,438-fold, and the recovery was 24% (Table 2). At the final size exclusion chromatography, aspirin hydrolytic activity was eluted asymmetrically with peak activity in fraction 42 (Figure 39A). SDS-PAGE was performed and followed by Coomassie blue staining for fraction 35 to 45 (Figure 39B). Arrows show bands co-located with the activity. Suspected bands were cut and identified by nano LC/MS (Figure 39C). PAFAH1B was identified with 5 peptides and 27% coverage as the only hydrolase in the material. Western blotting for PAFAH1B2 and PAFAH1B3 was performed in the gel filtration fractions where aspirin hydrolytic activity existed (Figure 39D). Both PAFAH1B2 and PAFAH1B3 were detected in the activity fractions and correlated with activity. In order to confirm type I PAFAH was an erythrocyte aspirin hydrolase, I overexpressed one of catalytic subunits of type I PAFAH ($\alpha 2$) and measured aspirin hydrolytic activity in the HEK 293T cells (Figure 40). Western blotting for PAFAH1B2 was performed to confirm overexpression. Aspirin hydrolytic activity was examined in the presence or absence of inhibitors in lysates of same number of overexpressed or normal cells. Aspirin hydrolytic activity of PAFAH1B2 overexpressed HEK 293T cells was found 6-fold more than that of control HEK 293T cells, which can be inhibited to control level by 2mM DTNB. 2mM MAFP was found to totally inhibit aspirin hydrolytic activity both in overexpressed or control HEK 293T cells. PAF hydrolytic activity was also examined

as above (Figure 41). It was found the same as aspirin hydrolytic activity. I also examined recombinant PAFAH1B2 for aspirin hydrolytic activity (Figure 42). 0.1µg, 0.2µg, and 0.4µg of recombinant PAFAH1B2 were incubated with 4mM aspirin in the presence of 40mM DTT at 37°C for 1 hour. Hydrolyzed aspirin increased in a concentration dependent manner. I also overexpressed PAFAH1B3 in HEK 293 cells, same results as PAFAH1B2 (Figure 43). Effects of NaF, a PAFAH I inhibitor, on purified erythrocytes aspirin hydrolase were examined (Figure 44). NaF inhibited aspirin hydrolytic activity of erythrocytes in a dose dependent manner. This result consisted with the inhibitory effects of NaF on PAF hydrolytic activity (Stafforini, Rollins et al. 1993). I also found a minor aspirin hydrolytic activity peak when I did the first DEAE chromatography (Figure 38A). This phenomenon was consistent with that showed by Ken Karasawa et al. , although they used pig erythrocytes as a enzyme source (Karasawa, Shirakura et al. 2005). I purified it as a single band in native PAGE, but several bands formed by SDS-PAGE (Figure 45). I found PAFAH1B2 as one of these bands by western blotting and identified all other bands as N-acylaminoacyl-peptide hydrolase (APEH) by mass spectrometry. I excluded APEH as an aspirin hydrolase by overexpression (Figure 46). APEH was overexpressed in HEK 293T cells, which confirmed by both western blotting and APEH activity (Figure 46A). However, no increase of aspirin hydrolytic activity was detected in the APEH overexpressed cells comparing with control cells (Figure 46B). It suggests that type I PAFAH accounts for aspirin hydrolytic activity of erythrocytes.

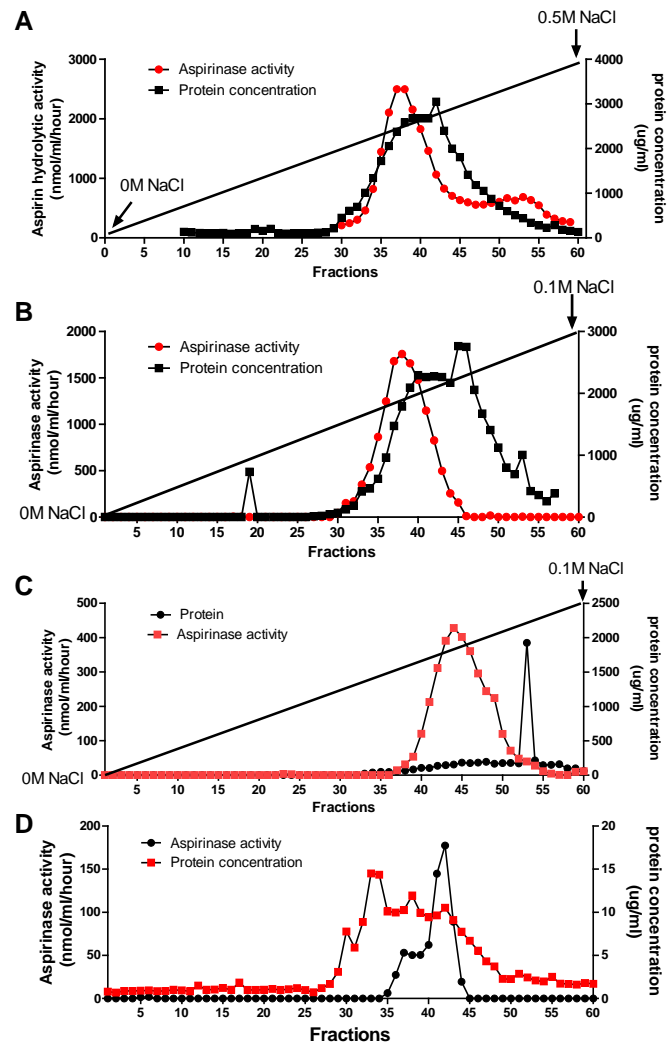


Figure 38. Elution profile of erythrocyte aspirin hydrolase purification. (A) 0-0.5 M NaCl linear gradient elution of EDAE chromatography. (B) 0-0.1 M NaCl linear gradient elution of EDAE chromatography. (C) 0-0.1 M NaCl linear gradient elution of HiTrap Q chromatography. (D) Sephacryl S-200 gel filtration chromatography.

Table 2

	Total protein (mg)	Total activity (nmol/hour)	Specific activity (nmol/mg/hour)	Purification (fold)	Recovery (%)
RBC lysate	10485.00	129339.65	12.33	1	100.00
DEAE binding	4100.00	99288.60	24.82	2	76.77
DEAE I	91.39	60012.00	656.66	53	46.40
DEAE II	41.56	37083.70	892.29	72	28.67
HiTrap Q FF	12.40	34260.00	2762.90	224	26.49
Sephacryl S-200	1.80	31924.01	17735.60	1438	24.68

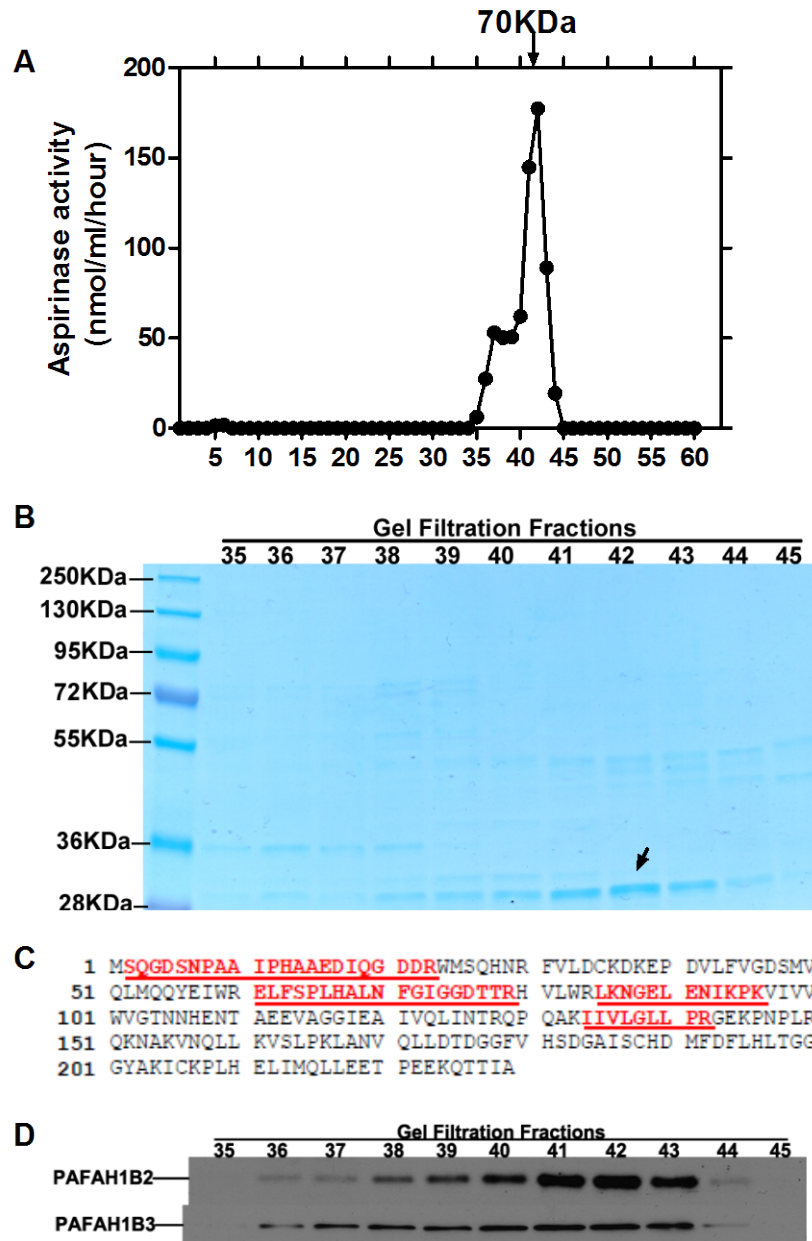


Figure 39. Type I Platelet-activating Factor acetylhydrolase is the primary erythrocyte aspirin hydrolase. (A) Aspirin hydrolytic activity was measured in gel filtration chromatography fractions. Maximal activity was eluted in fraction 41 to 43 where a size of 70 KDa located. (B) SDS-PAGE for gel filtration fractions (35-45). 10 μ l of each fraction was isolated by 4-15% gradient SDS-PAGE and followed by Commassie Blue staining. Arrow shows bands which were analyzed by mass spectrometry. (C) Results of mass spectrometry. PAFAH1B2 was identified by 5 peptides (underlined) with 27% coverage. (D) Western blotting for PAFAH1B2&3 in fraction 35 to 45.

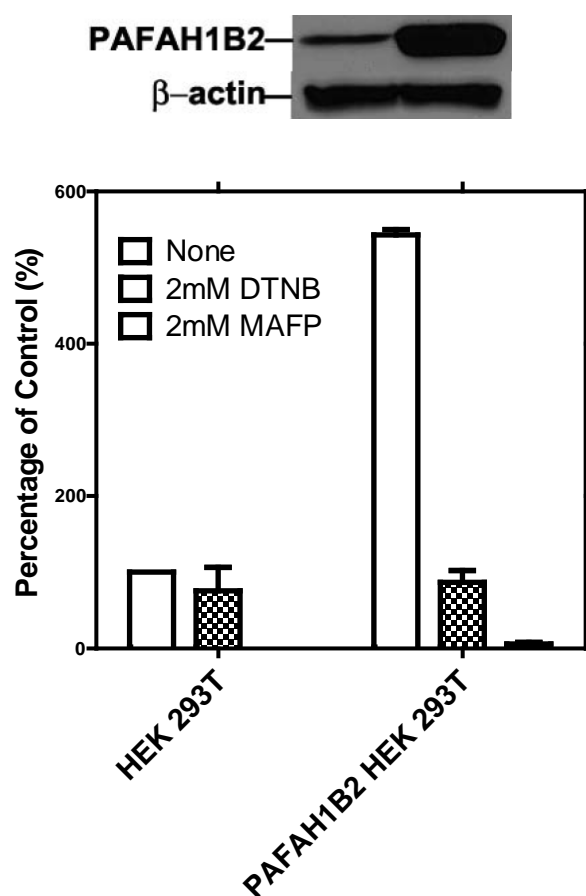


Figure 40. Overexpression of PAFAH1B2 increases aspirin hydrolytic activity of cell lysates. PAFAH1B2 was overexpressed in HEK 293K cells. Western blotting was performed to confirm PAFAH1B2 overexpression. Aspirin hydrolytic activity was examined in the cell lysates in the presence or absence of inhibitors. Data are present as mean \pm S.D., n=3.

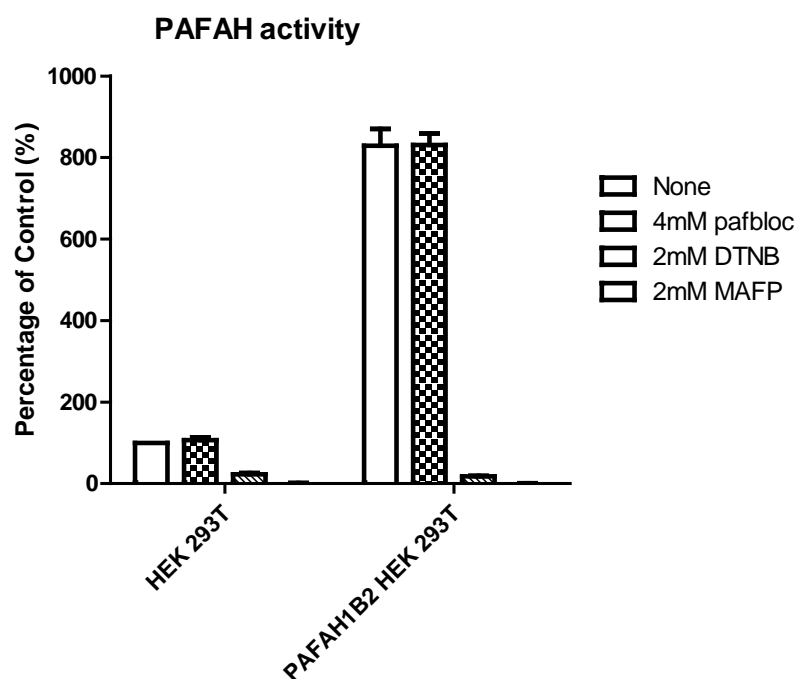


Figure 41. Overexpression of PAFAH1B2 increases PAF acetylhydrolase activity of cell lysates. PAFAH1B2 was overexpressed in HEK 293K cells. Western blotting was performed to confirm PAFAH1B2 overexpression. PAF acetylhydrolase activity was examined in the cell lysates in the presence or absence of inhibitors. Data are present as mean \pm S.D., n=3.

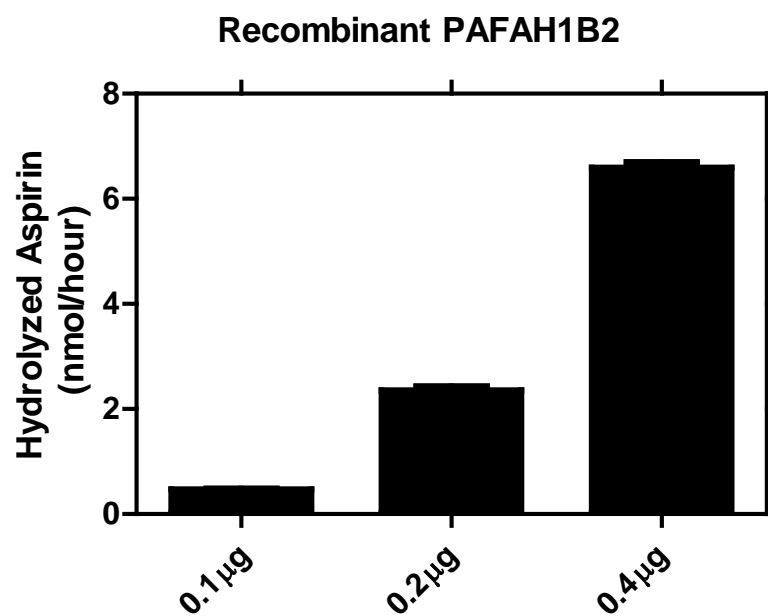


Figure 42. Recombinant PAFAH1B2 hydrolyzes aspirin. 0.1 µg, 0.2 µg, and 0.4 µg recombinant PAFAH1B2 were incubated with 4 mM aspirin at 37°C for 2 hours. Salicylic acid product was measured by RP-HPLC. Aspirin hydrolysis was found a dose dependent increase. Data are present as mean \pm S.D., n=3.

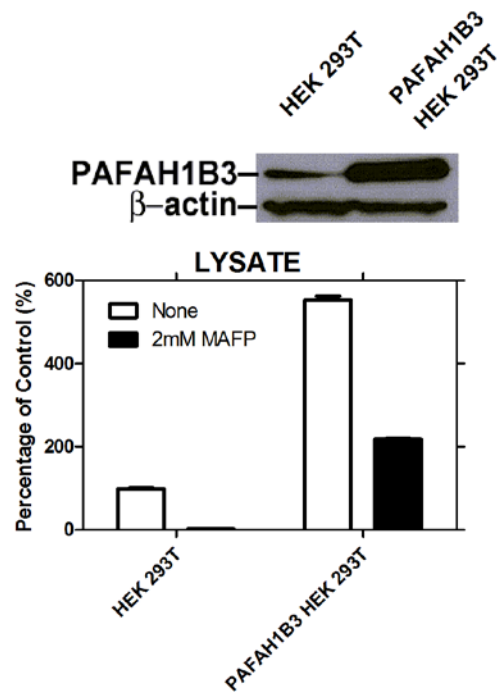


Figure 43. Overexpression of PAFAH1B3 increases aspirin hydrolytic activity of cell lysates. PAFAH1B3 was overexpressed in HEK 293K cells. Western blotting was performed to confirm PAFAH1B3 overexpression. Aspirin hydrolytic activity was examined in the cell lysates in the presence or absence of inhibitors. Data are present as mean \pm S.D., n=3.

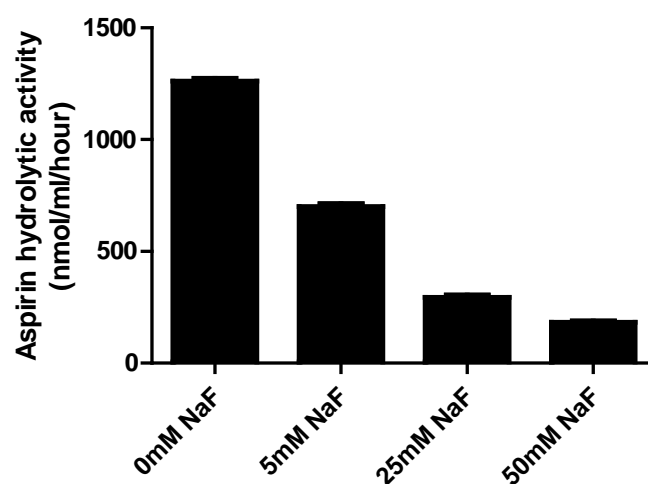


Figure 44. NaF inhibits aspirin hydrolytic activity of purified erythrocyte aspirin hydrolase. 4 mM aspirin was incubated with NaF of different concentration (0 mM to 50 mM). Aspirin hydrolytic activity was inhibited by NaF in a dose dependent manner. Data are present as mean \pm S.D., n=3.

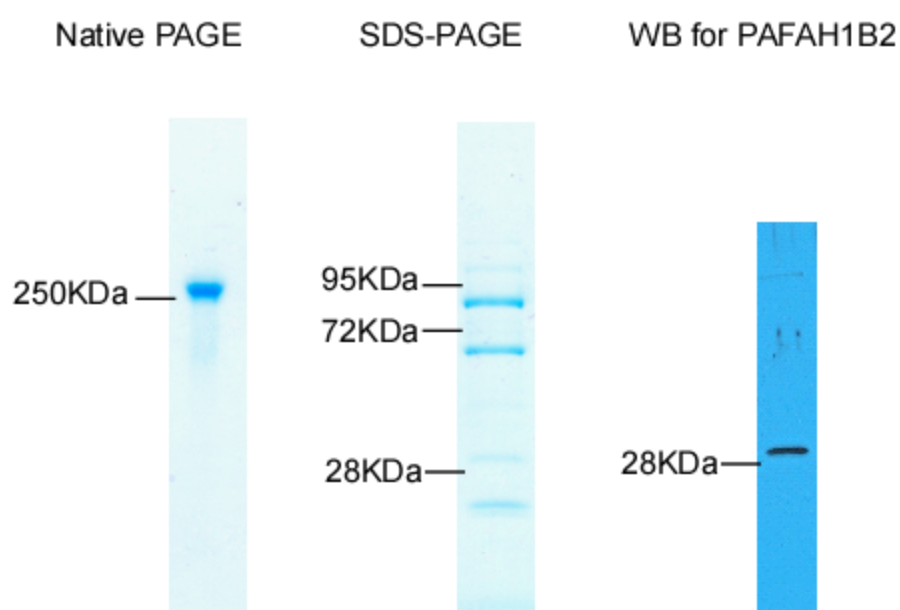


Figure 45. PAFAH1B2 was detected in purified minor part of erythrocyte aspirin hydrolytic activity. Minor part of aspirin hydrolytic activity from the first DEAE chromatography (Supplemental. Fig. 2A) was purified by cation exchange and gel filtration chromatography. Native PAGE after Coomassie blue staining showed a single band. The same amount of purified minor part of aspirin hydrolytic activity was subjected onto SDS-PAGE and several bands were separated and stained by Coomassie blue. PAFAH1B2 was detected by western blotting. All other bands were identified as APEH by mass spectrometry.

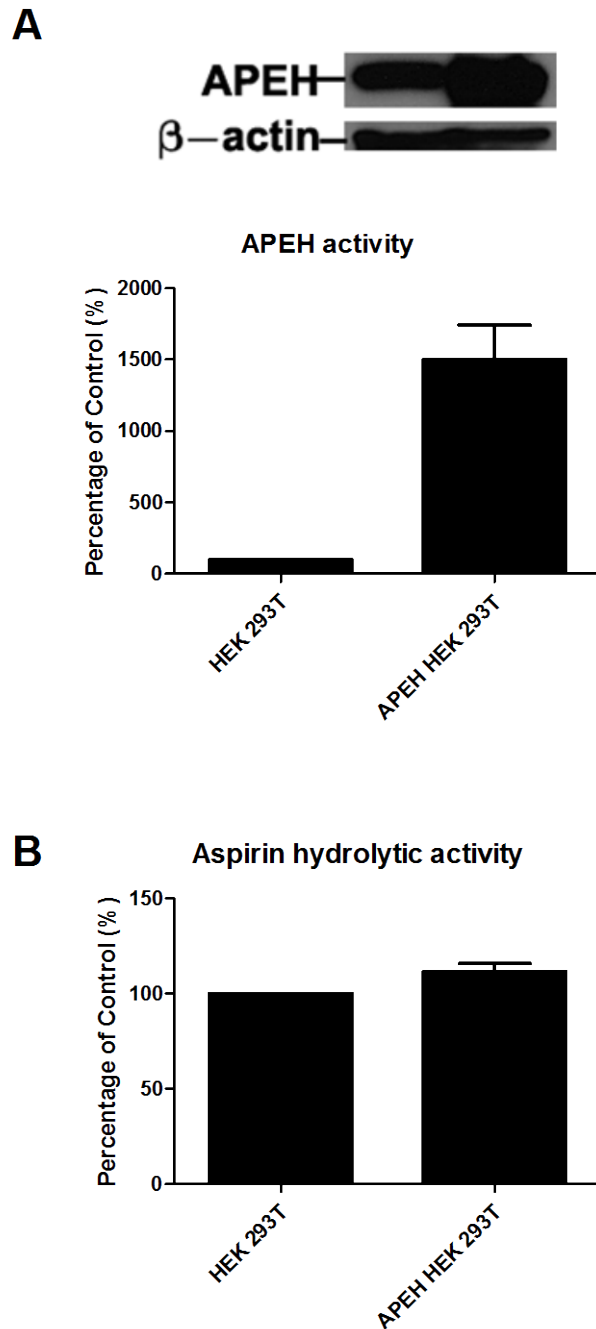


Figure 46. APEH does not have aspirin hydrolytic activity. (A) APEH was overexpressed in HEK 293T cells. Both western blotting for APEH and APEH activity assay were performed to confirm that APEH was overexpressed successfully. (B) Aspirin hydrolytic activity was examined in cell lysates of both APEH overexpressed and normal HEK 293T cells. No increased aspirin hydrolytic activity was found. Data are present as mean \pm S.D., $n \geq 3$.

4.2.4. Type I PAFAH hydrolyzes aspirin efficiently.

Effect of PAF on aspirin hydrolysis by type I PAFAH was examined. I verified that erythrocyte PAF acetylhydrolase was an appropriate candidate aspirin hydrolase by testing aspirin hydrolysis by PAFAH1B2 because, at least for PAF at low concentrations, the type I complex is more effective as the PAFAH1B2 homodimer or PAFAH1B2 and PAFAH1B3 heterodimer (Manya, Aoki et al. 1999; Karasawa, Shirakura et al. 2005) than the enzyme containing the PAFAH1B3 homodimer. Recombinant PAFAH1B2 effectively hydrolyzed aspirin, and its rate varied with substrate concentration that was well described by the Michaelis-Menten equation (Figure 47). The K_m was determined to be 1.13 ± 0.57 mM and its V_{max} was 21.4 ± 2.95 nmol/h/ μ g. The enzyme purified from erythrocytes displayed an apparent K_m for aspirin of 2.17 ± 0.65 mM with a corresponding V_{max} of 66.05 ± 7.18 nmol/h/ μ g, suggesting the recombinant material was not fully active. Butyrylcholinesterase is the most completely characterized soluble enzyme to hydrolyze aspirin, which hydrolyzed aspirin with a K_m of 2.78 ± 0.59 mM and a V_{max} of 56.36 ± 4.78 nmol/h/ μ g. I determined whether the acetyl group of PAF and aspirin were likely hydrolyzed in the same active site of the type I PAF acetylhydrolase, and tested the effect of PAF on aspirin hydrolysis by the purified erythrocyte enzyme and recombinant PAFAH1B2. I found that the short chain phospholipid inhibited hydrolysis of the xenobiotic aspirin substrate by both enzymes (Figure 48), and that this inhibition was competitive for both enzymes. The calculated K_i for PAF inhibition of aspirin hydrolysis for the purified PAFAH1B2 was 0.47 mM,

whereas that for recombinant PAFAH1B2 was 0.29 mM. This indicates a strong preference for PAF compared with aspirin by recombinant PAFAH1B2.

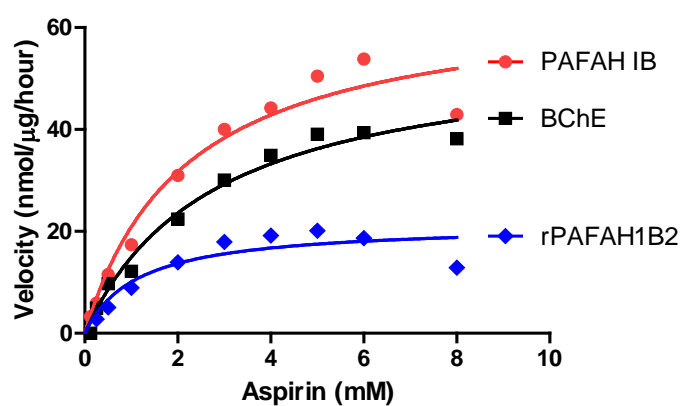


Figure 47. A calculated Michaelis-Menten curve of aspirin hydrolyase by nonlinear regression analysis. BChE, purified erythrocyte aspirin hydrolase, or rPAFAH1B2 were incubated with different concentration of aspirin at 37°C for 1 hour and followed salicylic acid quantification. K_m of BChE is 2.78 mM. K_m of PAFAH1B is 2.17 mM. K_m of rPAFAH1B2 is 1.17 mM. Data was analyzed by prism 4.

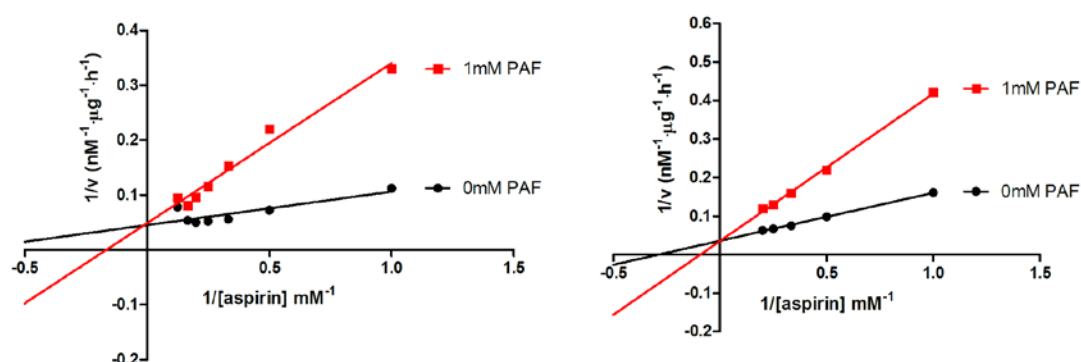


Figure 48. PAF competitively inhibits aspirin hydrolysis by erythrocyte aspirin hydrolase. Aspirin hydrolysis was measured in duplicates containing fixed amount purified type I PAFAH (400 ng) and varied aspirin (0.5–8 mM) in PBS buffer at pH 7.2 in the absence or presence of 1 mM PAF. Purified erythrocyte aspirin hydrolase (*right*) or recombinant PAFAH1B2 (*left*) was incubated with aspirin of different concentration in the presence or absence of PAF at 37°C for 2 hours followed by RP - HPLC measurement. Lineweaver–Burke plot was converted from Michaelis–Menten plot. K_i value of purified erythrocyte aspirin hydrolase is 0.47 mM; K_i value of rPAFAH1B2 is 0.29 mM. Data was analyzed by prism 4.

4.2.5. Erythrocyte Aspirin Hydrolysis Varies among Individuals.

The effectiveness of aspirin in inhibiting platelet function *ex vivo* varies among individuals and populations for unknown reasons (Patrono 2003; Adebayo, Williams et al. 2007; Fitzgerald and Maree 2007; Kotani, Kimura et al. 2010). I found that hydrolysis varied by over 2-fold when erythrocytes were prepared from 10 different donors (Figure 49A). Hydrolysis in lysates from a single donor, however, was reproducible (not shown). I assessed variation of type I PAF acetylhydrolase subunits in the blood of these donors to find that total PAFAH1B2 also varied among donors, whereas the PAFAH1B3 content was uniform (Figure 49B). Moreover, variation of PAFAH1B2, and not PAFAH1B3, content correlated with aspirin hydrolysis, suggesting the PAFAH1B2 containing type I PAF acetylhydrolase is the most effective aspirin hydrolase of erythrocytes, and blood.

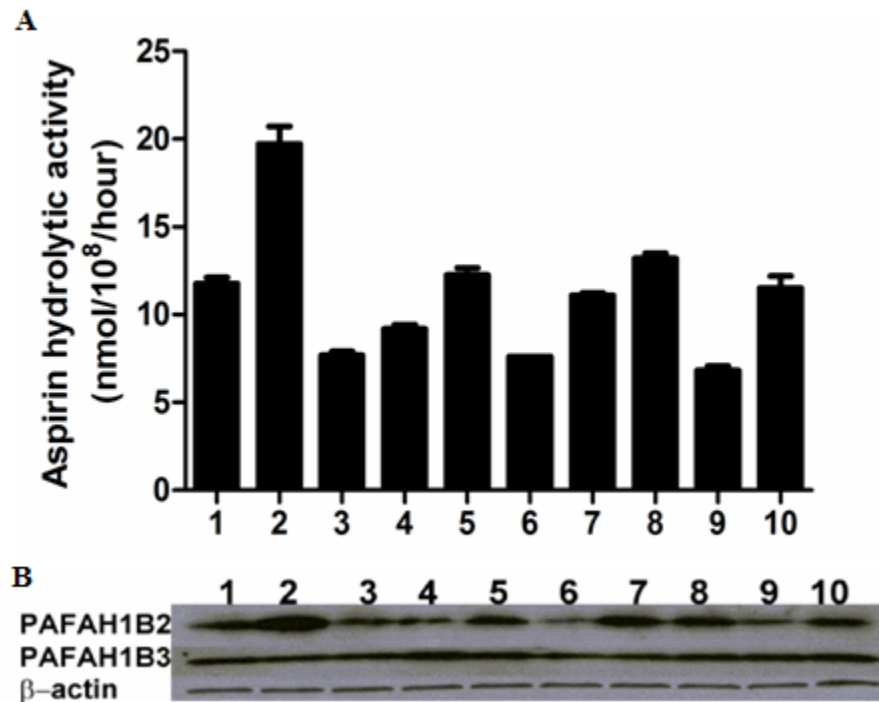


Figure 49. PAFAH1B2 and aspirin hydrolytic activity vary among donors. (A) Variation in the inter-individual rate of erythrocyte hydrolysis of aspirin. Aspirin hydrolytic activity was measured as described in Chapter II erythrocytes (10⁸) obtained from 10 random blood donors. Data are presented by mean \pm S.D., $n = 3$. (B) PAFAH1B2 varied among blood donors. PAFAH1B2 (*upper panel*), PAFAH1B3 (*lower panel*), or β -actin in samples from the donors in *panel A* were determined by immunoblotting as described under chapter II.

4.3. Discussion

In this chapter, I purified and identified erythrocyte aspirinase as type I PAFAH. I also determined its apparent K_m as 2.17 ± 0.59 mM using aspirin as a substrate. The K_i is 0.47 mM using 1 mM PAF as a competitive inhibitor. I discovered variation of erythrocyte aspirinase activity among individuals, which related to PAFAH1B2.

Aspirin hydrolysis was measured by RP-HPLC based quantifying salicylate product, which is more accurate than a spectrophotometer based method because the salicylate product of aspirin is not only derived from hydrolysis but also from acetylation. Erythrocyte aspirin hydrolase contributes more than half of aspirin hydrolysis activity in blood (Costello, Caruana et al. 1984). My data showed erythrocytes had a higher aspirin hydrolytic activity compared to plasma and contribute half or more to aspirin hydrolysis in whole blood. This, however, was only examined from a single donor, and the ratio of hydrolysis in plasma to cells may vary among donors. Albumin controls aspirin entering erythrocytes by reversibly binding and transporting this amphipathic molecule (Costello and Green 1987). Although albumin slowed aspirin hydrolysis in erythrocytes, it may increase the chance of plasma aspirin hydrolysis. Erythrocytes readily accumulate aspirin through band 3, an anion channel, from their environment (Ohsako, Matsumoto et al. 1993), and aspirin hydrolysis by erythrocyte lysates exceeded that by plasma enzymes. Previously, I identified the plasma enzymes as butyrylcholinesterase and a second equally effective, unidentified activity. The activity of aspirin hydrolysis by both activities varied

significantly in donor plasma, and the effectiveness of erythrocyte lysates in hydrolyzing aspirin also varied by several fold among donors. This difference in the ability to rapidly clear vascular aspirin by hydrolysis may be a reason of the apparent variation in the effectiveness of aspirin prophylaxis, aspirin resistance, among populations and individuals (Maree, Cox et al. 2007; Patrono and Rocca 2007).

I identified a single major activity of aspirin hydrolytic activity in erythrocytes as type I PAF acetylhydrolase by purifying the activities from erythrocyte lysates that hydrolyze aspirin. I discovered this consists of the PAF acetylhydrolase type I complex, but also apparently a second activity (ies) isolated by a larger protein or complex resolved by size separation whose identity was proved as PAFAH1B2. The PAF acetylhydrolase complex did not migrate as a single entity, but the type I PAFAH containing PAFAH1B3 homodimers differs modestly compared to the complex containing PAFAH1B2 homodimers (Karasawa, Shirakura et al. 2005). The two different activity complexes of type I PAFAH were also found by Karasawa, K et al. They purified type I PAFAH and also found two activities, although they used PAF as a substrate to purify the activity from pig erythrocytes (Karasawa, Shirakura et al. 2005). And they found that it was not possible to purify the minor activity (they called it as fraction X) to homogeneity. Their findings supported our results that purified minor erythrocyte aspirin hydrolytic activity was found a complex with abundance of APEH, and PAFAH1B2 was also detected in it by western blot. And this kind of complex was also found in platelets and neutrophils.

Aspirin hydrolytic activity is largely a property of erythrocytes, which quickly internalizes it through prior to intracellular hydrolysis (Ohsako, Matsumoto et al. 1993). Diisopropylfluorophosphonate, like the methyl arachidonoyl fluorophosphonate used here, repressed erythrocyte hydrolysis of aspirin consistent with membrane-bound acetylcholinesterase (Costello and Green 1982). Costello, P.B. et al. purified aspirin hydrolytic activity about 900-fold from a half saturated $(\text{NH}_4)_2\text{SO}_4$ solution of erythrocyte lysates by DEAE sephacel chromatography. A 95 KDa protein was isolated by SDS-PAGE as erythrocyte aspirin hydrolase, which is sulfhydryl group dependent but different from the nonspecific type D esterases (Costello and Green 1983). Type I PAFAH is a sulfhydryl group dependent enzyme and has a molecular weight of 29 or 30 KDa but not 95 KDa. I cannot reconcile our identification of 30 and 29 kDa PAF acetylhydrolases with this apparent molecular weight, but when nondenatured the type I PAF acetylhydrolase exists as a trimer of either homo- or heterodimers of 29 and 30 kDa catalytically active subunits together with a non-catalytic subunit LIS1 of 45 kDa (Hattori, Adachi et al. 1994). Costello, P.B. et al. most probably purified this as their erythrocyte aspirin hydrolytic activity. Recombinant PAFAH1B2 or lysates of HEK 293 cells transiently transfected with this gene hydrolyzed aspirin, aspirin and PAF competed with one another for hydrolysis, and hydrolysis was appropriately blocked by DTNB and diisopropylfluorophosphonate, established inhibitors of erythrocyte PAF acetylhydrolase (Stafforini, Rollins et al. 1993). These data all give support to type I PAFAH as an aspirin hydrolase.

Over 100 million tons of aspirin is consumed per year (Warner and Mitchell 2002) as anti-pyretic and anti-inflammatory therapeutics, and as a prophylaxis for major cardiovascular events. The pharmacokinetics show that ingested aspirin has only a short half-life in the circulation of approximately 20 min (Needs and Brooks 1985; Higgs, Salmon et al. 1987). The primary medical target of the acetyl functional group of acetylsalicylic acid is type I cyclooxygenase (prostaglandin H-synthase) (Roth, Stanford et al. 1975) of platelets that irreversibly inhibits production of the prostaglandin H₂ precursor for platelet thromboxane formation and thereby sharply reduces platelet reactivity (Smith, DeWitt et al. 2000). Hydrolysis of aspirin, and hence control of circulating aspirin levels, varies greatly among individuals and is increased in individuals with inflammatory phenotypes (Williams, Asad et al. 1987; Akopov, Grigorian et al. 1992; Puche, Gomez-Valverde et al. 1993; Gresner, Dolnik et al. 2006; Kotani, Kimura et al. 2010). As a subsequence, efficiency of aspirin on anti-platelet also varies among individuals accordingly.

Type I PAF acetylhydrolase was initially purified from bovine brain (Hattori, Arai et al. 1993) and determined to be a unique esterase (Hattori, Adachi et al. 1994; Ho, Swenson et al. 1997) unrelated to the other two members of the group VII phospholipase A₂ family (Six and Dennis 2000). Subsequently, red blood cells were found to richly express the type I PAF acetylhydrolase (Karasawa, Shirakura et al. 2005). This identification was consistent with the low molecular weight of the enzyme purified from erythrocytes found to be sensitive to NaF, DTNB, and diisopropylfluorophosphonate (Stafforini, Rollins et al. 1993). Physiological function

of type I PAFAH is still under exploration. PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine), a lipid mediator, is believed its physiological substrate, since PAF hydrolyzing activity was used to purify type I PAFAH. PAFAH1B2 was showed as a serine esterase with an activity serine 47 residue and had some homology to PAF receptor (Hattori, Adachi et al. 1994). Animal studies showed PAFAH1B involved in spermatogenesis (Koizumi, Yamaguchi et al. 2003; Yan, Assadi et al. 2003) and neuronal migration (Manya, Aoki et al. 1998).

Aspirin as an artificial substrate of type I PAFAH may affect those physiological functions of people with long term aspirin medication. However, the type I PAF acetylhydrolase serves no known role in erythrocytes because erythrocytes neither synthesize the phospholipid autocoid PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) nor readily accumulate it from their environment (Chen, Yang et al. 2007). This is critical because while other PAF acetylhydrolases act on oxidized phospholipids (Stremmler, Stafforini et al. 1989), the type I enzyme is tightly constrained to acetyl hydrolysis (Ho, Sheffield et al. 1999). Genetic ablation of either the PAFAH1B2 or PAFAH1B3 gene does not identify a physiologic role for this enzyme complex because neither displays defective erythrocyte function (Koizumi, Yamaguchi et al. 2003). Still, the crystal structure of this esterase, like the structural homolog in *Streptomyces exfoliates* (Wei, Swenson et al. 1998), shows that only acetyl esters are accommodated in the active site (Ho, Swenson et al. 1997; Ho, Sheffield et al. 1999), and may allow access for the acetyl functional group of aspirin.

Aspirin has been a popular and effective drug over the past century or even now, but the agents responsible for its rapid turnover in the circulation have not been identified. Purification shows the type I PAF acetylhydrolase esterase complex is a major component of aspirin hydrolysis activity in blood. The ability to accept an acetyl functional group is likely important in acceptance of aspirin as a substrate by this enzyme complex, but the two catalytic subunits have distinct requirements for the glycerophospholipid of acetylated phospholipids (Manya, Aoki et al. 1999) so acceptance of aspirin must involve more than simple recognition of the acetyl group. Individuals have different abilities to hydrolyze aspirin in plasma and in erythrocytes, the major contributors to aspirin turnover in blood, and this may be a component of the variation in aspirin effectiveness, or aspirin “resistance” (Marshall, Williams et al. 1997; Mueller, Salat et al. 1997; Stejskal, Proskova et al. 2004; Tantry, Bliden et al. 2005).

In conclusion, type I PAFAH is the aspirin hydrolase of erythrocytes. Using aspirin as a substrate, kinetic properties of type I PAFAH have been characterized. Aspirin hydrolytic activity of erythrocytes varies among individuals, which related with PAFAH1B2.

CHAPTER V

5. ELUCIDATION OF THE RELATIONSHIP BETWEEN ASPIRIN

HYDROLASES AND ASPIRIN EFFICACY

5.1. Introduction

In Western societies, the leading causes of death and the increasing costs of health care are cardiovascular diseases (CVD), especially myocardial infarction (Sans, Kesteloot et al. 1997). It was reported that aspirin (300 mg/day) reduced total mortality in patients one year after myocardial infarction by 25.7%, although it was not statistically significant at conventional levels ($p > 0.05$) (Elwood, Cochrane et al. 1974). However, it first suggested that administration of aspirin regularly may prevent a second heart attack. Nowadays aspirin is widely used as a prophylaxis reagent of cardiovascular disease, which is due to the major effect of aspirin, anti-platelet function. This function is accredited to the acetyl group of aspirin which irreversibly acetylates cyclooxygenase-1 (COX-1) at its 530 serine residual to inhibit thromboxane A₂ production (Szczeklik, Gryglewski et al. 1979). Preexisting risk factors, such as atherosclerotic alterations of the vessel wall, mainly determine the possibility of thrombotic events, which may through hyper reactive platelets (Gawaz

2004). As we know, active platelets produce lots of thromboxane. By measuring levels of plasma thromboxane in circulation, people found that platelets play important roles in unstable angina (Fitzgerald, Roy et al. 1986). Aspirin inhibits platelet activation by inhibiting formation of thromboxane, which will benefit those diseases. But 5–45% of patients with unstable angina and 5–65% of patients with stroke (McKee, Sane et al. 2002; FitzGerald 2003; Wang, Aucoin-Barry et al. 2003; Alberts, Bergman et al. 2004; Mason, Jacobs et al. 2005; Sztrika, Sas et al. 2005) are considered aspirin “resistant”. This makes aspirin impotence, although it an idea prevention medicine of heart diseases.

However, the benefit effects of aspirin on CVD was thought has nothing to do with inhibition of thromboxane (Bertele, Tomasiak et al. 1982), because thromboxane inhibiting effects are only detectable when high dose of aspirin is medicated (Undas, Brummel et al. 2001), as well as increased fibrinolytic activity and anti-inflammatory effects (Cattaneo 2004). High-dose aspirin was reported better than low-dose aspirin in stroke prevention effect (Tohgi, Konno et al. 1992), which was believed to be due to reduced response of platelets to aspirin in these survived patients after the acute heart attack (Grote Meyer 1991). So dosing of aspirin is pivotal and the mechanism of aspirin is complicated. Variation of response to aspirin in these patients (Helgason, Bolin et al. 1994) was confirmed by Gurbel et al. (Gurbel, Bliden et al. 2007). So there must be a certain group of patients in Gurbel’s study whose platelets are insufficient inhibited by the same dose of aspirin (Schrör, Weber et al. 2006).

No matter what the mechanism of aspirin is, anti-platelet function of aspirin is well documented and dosing of aspirin is critical. Blood aspirin hydrolases are important in controlling exposure time of platelet into aspirin. I have shown that significant aspirin hydrolytic activity variation in plasma as well as in erythrocytes. I identified blood aspirin hydrolases as BChE and type I PAFAH. How these aspirin hydrolases modulate anti-platelet function of aspirin is still undefined. I investigated effects of both plasma aspirin hydrolase and erythrocyte aspirin hydroalse on anti-platelet function of aspirin and found variation of aspirin hydrolytic activity varied aspirin efficacy in vitro.

5.2. Results

5.2.1. Anti-platelet function of aspirin is dose dependent.

Aspirin irreversibly acetylates cyclooxygenase-1 of platelets which inhibits thromboxane A₂ production to inhibit platelet activation. When platelets are activated, they will aggregate and release other agonists such as ADP, a potent agonist, to recruit other platelets as well as ATP for other cells of the blood (Sung, Young et al. 1985; Greenberg, Di Virgilio et al. 1988). I investigated concentration effect of aspirin on platelet aggregation (Figure 50). 10^8 washed platelets in 500 μ l HBSS buffer were incubated with aspirin of different concentration (25 μ M, 33 μ M, and 50 μ M) at 37°C for 5 min and followed stimulation by 5 μ g/ml collagen. Aspirin inhibited platelet aggregation in a concentration dependent manner with 33 μ M aspirin being the lowest concentration to inhibit platelet aggregation.

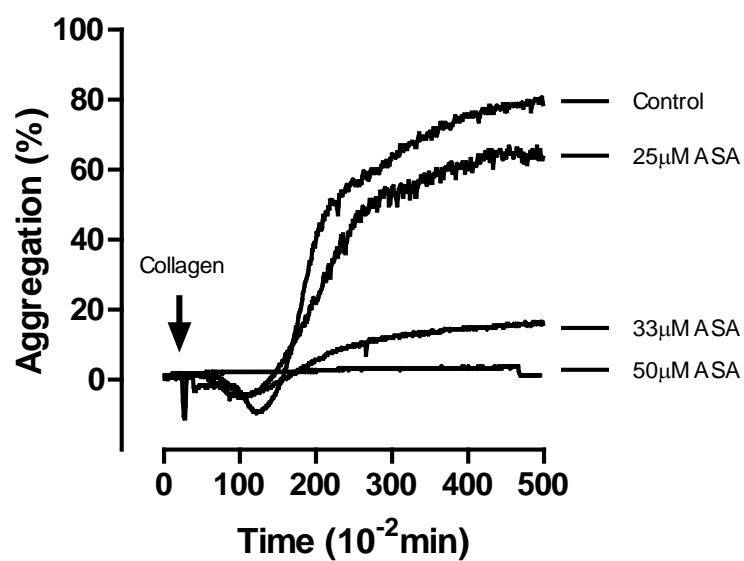


Figure 50. Anti-platelet effect of aspirin is dose dependent. Different concentration of aspirin treated washed platelets (10^8) were stimulated by 5 $\mu\text{g/ml}$ collagen. Platelet aggregation was recorded by optical aggregometer. $n=8$.

5.2.2. Variation of plasma aspirin hydrolytic activity modulates anti-platelet function of aspirin.

Since we knew that significant variation of plasma aspirin hydrolytic activity existed in population, I also investigated the effect of the variation on anti-platelet function of aspirin. I first chose platelet poor plasmas (PPP) with different aspirin hydrolytic activities. PPP of donor 3 had high level of aspirin hydrolytic activity; PPP of donor 6 had low level of aspirin hydrolytic activity (Figure 27C). I pre-incubated 33 μ M aspirin with these PPP at 37°C for 30 min and then performed platelet aggregation inhibition experiment (Figure 51). PPP of donor 3, which had high level of aspirin hydrolytic activity, impaired more anti-platelet function of aspirin than that of PPP of donor 6, which had low level of aspirin hydrolytic activity. And I also knew that variation of plasma aspirin hydrolytic activity is due to BChE. So I examined effect of BChE on anti-platelet function of aspirin. I pre-incubated 33 μ M aspirin with 2 μ g BChE at 37°C for 30 min and followed stimulation by 5 μ g/ml collagen (Figure 52). Comparing with control, anti-platelet effect of aspirin was impaired by pre-incubation with BChE. It suggests that variation of plasma aspirin hydrolytic activity can modulate anti-platelet function of aspirin.

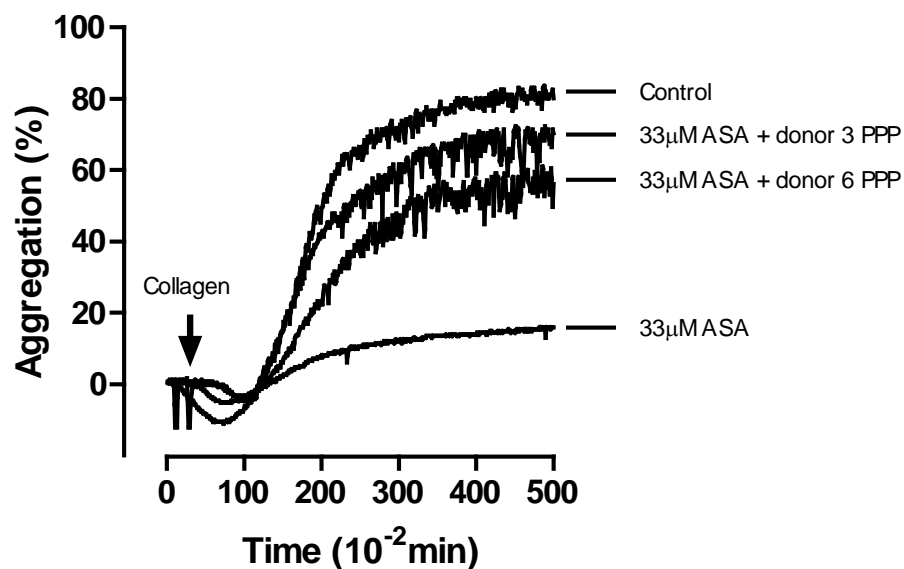


Figure 51. Plasma aspirin hydrolytic activity modulates anti-platelet function of aspirin. Aspirin was pre-incubated with plasma (400 μ l) of different donors with different aspirin hydrolytic activity at 37°C for 30 min. 10⁸ washed platelets in 100 μ l were added into the plasma. After 5min incubation, platelets were stimulated by 5 μ g/ml collagen. n=3.

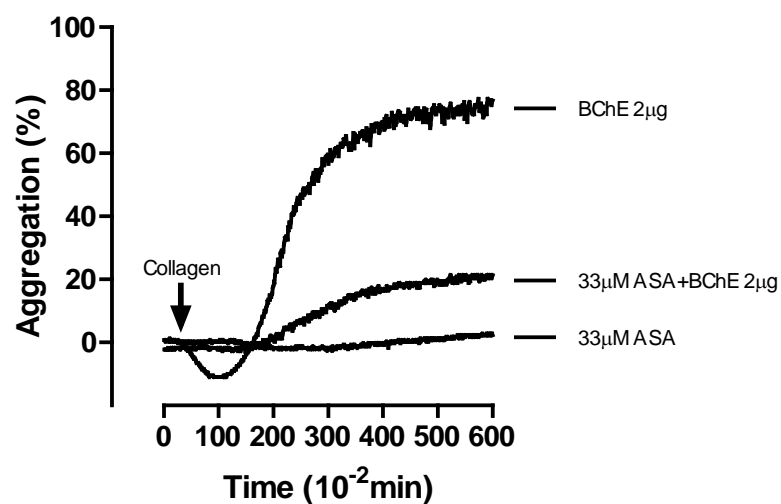


Figure 52. BChE suppresses anti-platelet function of aspirin. Aspirin was pre-incubated with 2 μg BChE at 37°C for 30 min. 10^8 washed platelets in 100 μl were added into the plasma. After 5min incubation, platelets were stimulated by 5 $\mu\text{g/ml}$ collagen. $n=3$.

5.2.3. Erythrocyte aspirin hydrolase contributes aspirin inactivation.

Erythrocytes as a major player of blood aspirin hydrolytic activity are also found variation among population but not that much as that of plasma (Figure 36). I examined effect of erythrocytes on anti-platelet function of aspirin. I found that the major effect of erythrocytes was due to aspirin hydrolase. Erythrocytes quickly inactivated aspirin and reduced its anti-platelet function. And this aspirin inactivating effect by erythrocytes can be inhibited by 50 mM NaF, which inhibits type I PAFAH. Aspirin was pre-incubated with 1 μ l washed erythrocytes for 30min at 37°C or without pre-incubation and then the erythrocytes and aspirin mixture was added to 108 washed platelets (450 μ l) for another 5 min which the aspirin would have been at a final concentration of 50 μ M. Platelets were stimulated by 5 μ g/ml collagen (Figure 53). 30 min incubation with erythrocytes totally eliminated 50 μ M aspirin's anti-platelet function. NaF inhibited the erythrocyte effect on aspirin's anti-platelet function (Figure 54) when erythrocytes were pretreated with the type I PAFAH inhibitor, 50 mM NaF, for 30 min. Aspirin incubated with NaF treated erythrocytes still showed 20% platelet inhibition ability comparing to that of normal erythrocytes. Aspirin hydrolytic activity was examined in NaF treated erythrocytes. Comparing to normal erythrocytes, aspirin hydrolytic activity of 50 mM NaF treated erythrocytes was found inhibited by 60%, which suggested negative modulation effect of erythrocytes on aspirin's anti-platelet function was due to its aspirin hydrolytic activity.

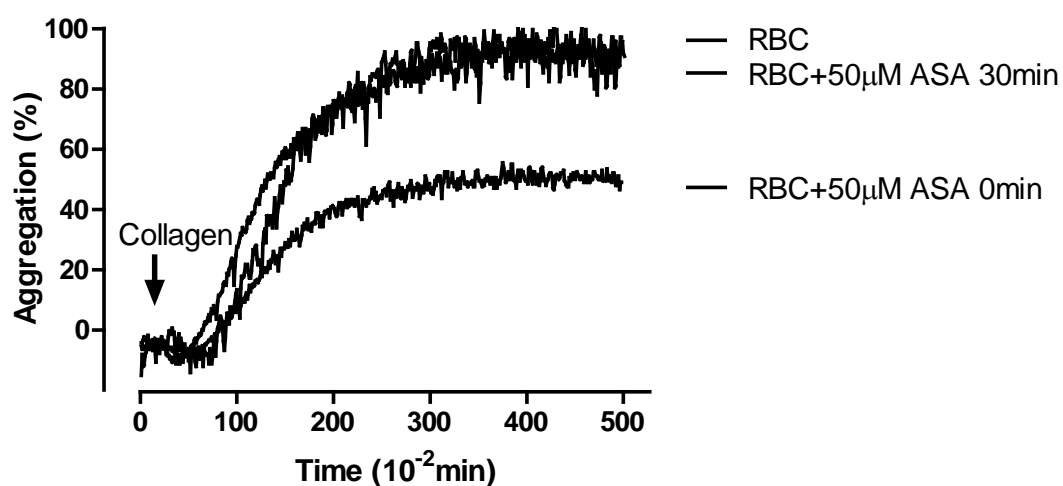


Figure 53. Erythrocytes suppress anti-platelet function of aspirin. 1 μ l erythrocytes were incubated with aspirin at 37°C for 30 min and then were put into washed platelets (10^8 in 450 μ l) for another 5min. Platelets were stimulated by 5 μ g/ml collagen.

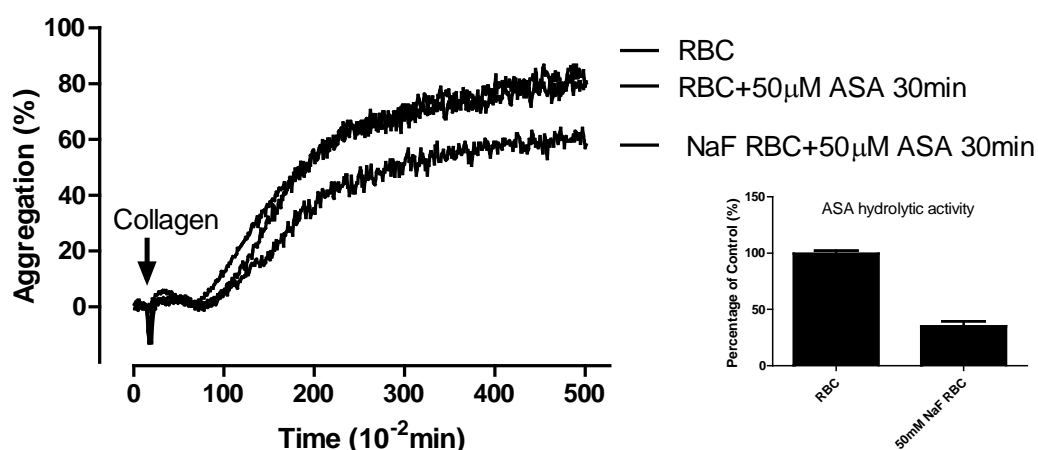


Figure 54. Aspirin hydrolase of erythrocytes is important in suppressing anti-platelet function of aspirin. Erythrocytes were treated with 50 mM NaF, which inhibited 60% of their activity. 1 μ l NaF treated or normal erythrocytes were incubated with aspirin for 30 min and then were added into washed platelets (10^8 in 450 μ l) for another 5 min. Platelets were stimulated by 5 μ g/ml collagen.

5.2.4. Erythrocytes reduce aspirin inhibition of platelet thromboxane B₂ production.

In order to make sure that the negative effect of erythrocyte on aspirin's anti-platelet function is because of aspirin hydrolysis. TxB₂, a metabolite of TxA₂, was examined. Production of thromboxane B₂ in platelets was reduced by 25 μM aspirin, but abolished by 50 μM aspirin (Figure 55, comparison 1). Erythrocytes neither made thromboxane B₂, nor suppressed its production by collagen-activated platelets. However, erythrocytes greatly reduced the effect of aspirin on platelet thromboxane B₂ production: erythrocytes preincubated with aspirin reduced the effectiveness of this drug and allowed platelet synthesis of the prostanoid to recover (Figure 55, comparison 2). Aspirin incubated with erythrocytes for 0 time was inhibitory, but aspirin preincubated with erythrocytes for 30 min was significantly less effective in blocking platelet thromboxane B₂ production (Figure 55, comparison 3). In fact, aspirin preincubated with erythrocytes was almost completely ineffective as a platelet inhibitor. NaF addition to erythrocytes along with aspirin reduced the inhibitory effect of erythrocytes on aspirin, and again allowed aspirin to inhibit platelet thromboxane B₂ production (Figure 55, comparison 4).

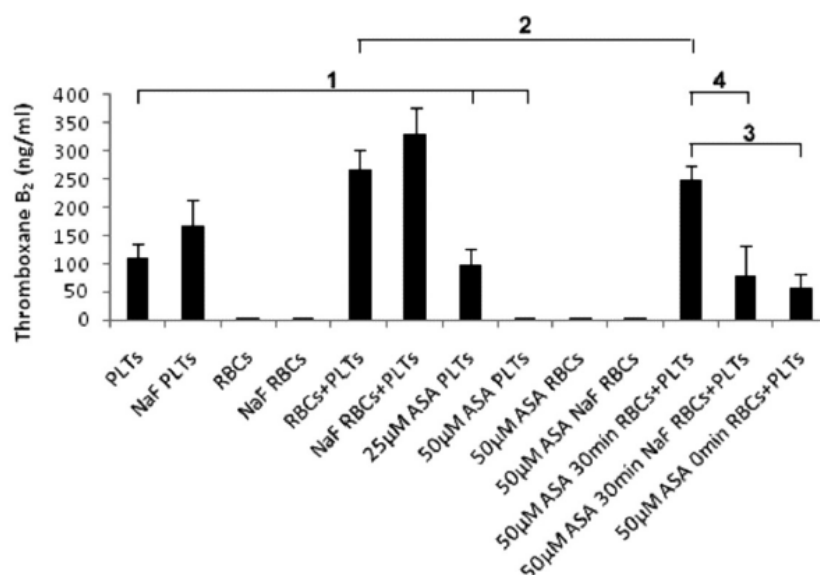


Figure 55. Erythrocytes reduce aspirin inhibition of platelet thromboxane B₂ production. Platelets were stimulated by 5 µg/ml of collagen after the stated treatments. Supernatants were collected and thromboxane B₂, the stable metabolite of thromboxane A₂, was measured by enzyme-linked immunoassay. Numbered comparisons are defined in the text and the data are present as mean ± S.D., $n \geq 3$.

5.3. Discussion

Aspirin has significantly inhibits platelet activation. This effect occurs quickly and depends on the dose of aspirin. Blood aspirin hydrolases, whether in plasma or within erythrocytes, play an important role in controlling anti-platelet function of aspirin. I elucidated the relationship between blood aspirin hydrolases and anti-platelet function of aspirin by *in vitro* experiments. My work showed that variation of blood aspirin hydrolytic activity can vary significantly.

Aspirin is calculated to increase to 44 μM in plasma one minute after a single 200 mg dose, with a real concentration of 13 μM measured 5 minutes after the oral dose (Higgs, Salmon et al. 1987). I found that 33 μM aspirin effectively suppressed washed platelet aggregation and that this inhibition was not linear in this immediate range, as previously found (FitzGerald, Maas et al. 1983). Platelets experiencing aspirin in plasma were less efficiently inhibited than in its absence, and this loss of potency was most pronounced in plasma of an individual with high butyrylcholinesterase activity and conversely was less in plasma of a donor with low activity. These results indicate that variation of plasma butyrylcholinesterase occurs over a range relevant to physiologic aspirin concentrations.

Platelet function was usually measured by light transmission aggregometer and platelets were stimulated by one particular agonist, collagen. There are few erythrocytes in many platelet function assays, which overlook effect of erythrocytes on platelet function and on aspirin's anti-platelet function. This may not capture or

reflect the real episode of aspirin's anti-platelet function. Erythrocytes markedly increase platelet reactivity (Santos, Valles et al. 1997) and reduce the antiplatelet actions of aspirin (Valles, Santos et al. 1998). Taking into consideration of possible effects of erythrocytes in blood on aspirin, erythrocyte aspirin hydrolase may influence efficacy of aspirin on the platelet-dependent hemostatic process. I also found that erythrocytes impaired aspirin's anti-platelet function by hydrolyzing aspirin.

Aspirin is a widely used drug to prevent cardiovascular and thrombotic diseases. However, in recent decade more cases of aspirin treatment failure drew our attention, which was so called "aspirin resistance". Aspirin "resistance" is a considerable clinical concern which describes phenomenon such as a reduced anti-platelet activity of aspirin or lacking benefit from aspirin. Aspirin resistance in population was reported proximately 8-45% of total aspirin medication cases (Grottemeyer 1991; Grottemeyer, Scharafinski et al. 1993; Helgason, Tortorice et al. 1993; Gum, Kottke-Marchant et al. 2001). Many reasons are proposed to explain "aspirin resistance", which makes it a very random event (Fontana, Nolli et al. 2006; Gurbel, Bliden et al. 2007) and still a matter of debate. One of pharmacokinetic reasons, insufficient bioavailability, could be more reasonable and fundamental for "aspirin resistance" because of aspirin's short half-life (15-20 min) and low dose usage (75-81 mg/day). Insufficient bioavailability is always a reason for less efficacy of any drug, including aspirin. Phase I biotransformation metabolism of aspirin in blood remains undefined. Widely believed so-called non-specific esterases catalyze

aspirin hydrolysis without further explanation. Understanding aspirin metabolism will contribute to improving its bioavailability, and help to reduce aspirin “resistance”. I identified blood aspirin hydrolases and characterized the role in anti-platelet function of aspirin. Aspirin administered at a small dose is more prone to subject a possibly low bioavailability, which was supported by the fact that a plain preparation of aspirin is more effective over an enteric-coated preparation of it (Cox, Maree et al. 2006). In addition, aspirin has a very short half-life about 20 min which may be shorter in low dose aspirin medication. This means newly produced platelets during the intervals of medication will still have the active COX-1 and synthesize bioactive TxA_2 to trigger platelet activation. High aspirin hydrolytic activity reduces intact aspirin access to COX-1 in platelets. My findings reveal that significant variation of blood aspirin hydrolytic activity significantly modulates anti-platelet function or ability of aspirin.

CHAPTER VI

6. CONCLUSIONS AND DISCUSSIONS

Blood aspirin hydrolases were purified, identified, and characterized. RP-HPLC analysis of the product salicylic acid with an internal standard showed plasma hydrolysis of aspirin varied 12-fold variation among 2,275 individuals. Genome-wide association analysis using serum aspirin hydrolytic activity from 2,275 individuals showed a genetic component to aspirin hydrolytic variation, and that only BChE significantly associated to aspirin variation. However, plasma from an individual with an inactivating point mutation in BChE effectively hydrolyzed aspirin. A non-BChE aspirin hydrolase was found in plasma, which can be distinguished from BChE by procainamide and oxidized ATP. Erythrocyte aspirin hydrolase was purified by 1400-fold and type I PAF acetylhydrolase was identified as a candidate aspirinase by mass spectrometry. Recombinant PAFAH1B2 and PAFAH1B hydrolyzed aspirin and aspirin was effectively hydrolyzed in cells ectopically expressing PAFAH1B2 or PAFAH1B3. Oxidized ATP inhibits recombinant PAFAH1B2, and it reduces aspirin hydrolysis in plasma. Type I PAFAH also accounts for non-BChE plasma aspirin hydrolytic activity. It is the first time that blood aspirin hydrolases have been identified and characterized. The relationship between blood aspirin hydrolases and

aspirin efficacy was investigated. Both plasma and erythrocytes significantly modulated aspirin efficacy, and they varied in parallel with aspirin hydrolytic activity. These findings may explain one cause of the phenomenon of aspirin resistance. These also provided personalized medicine of aspirin valuable data.

Aspirin prophylaxis suppresses major adverse cardiovascular events, but its turnover in blood is rapid, which limits its inhibition effects on platelet cyclooxygenase and thrombosis. Attention has focused on a phenomenon of aspirin ineffectiveness where not all individuals or populations appear to receive the full prophylactic or therapeutic benefits of aspirin (Williams, Asad et al. 1987; Akopov, Grigorian et al. 1992; Puche, Gomez-Valverde et al. 1993; Gresner, Dolnik et al. 2006; Kotani, Kimura et al. 2010). Inter-individual variability of platelet responses to aspirin clinically presents as treatment failure, and this increasing clinical phenomenon is frequently named aspirin “resistance”. The physiologic basis for this aspirin resistance is undefined, and even whether the phenomenon is real or documentable is debated (Patrono and Rocca 2007). Despite these clinical and laboratory issues, variation in the time aspirin circulates will affect its ability to inactivate cyclooxygenase 1 and inhibit platelet function. Aspirin turnover limits its effectiveness, so the enzyme(s) responsible for this hydrolysis is important. Aspirin hydrolysis was not subjected to natural selection, and so must be a property of enzymes selected in other ways. Accordingly, several esterases have the ability to hydrolyze aspirin that include uridine diphosphate glucuronyl transferase (Bigler, Whitton et al. 2001; Chan, Tranah et al. 2005), carboxylesterase 2 (Mentlein and Heymann 1984; Yamaori, Fujiyama et

al. 2006), and undefined activities distinct from cholinesterases and nonspecific carboxyesterases (Ali and Kaur 1983). However, the enzymes that actually contribute to aspirin turnover in blood need to be defined to better understand aspirin resistance.

In my thesis, I defined blood aspirin hydrolases by purifying them, and I found large variation of this activity among individuals. This may be one of many possible reasons that explain aspirin “resistance”. From point of pharmacokinetic view, insufficient bioavailability, could be more reasonable and fundamental to explain “aspirin resistance” because of aspirin’s short half-life (15-20min) and low dose usage (75-81mg/day). Higher aspirin hydrolytic activity would reduce its bioavailability. Clinical evidence is that a fixed lower dose aspirin was not enough to completely inhibit platelet aggregation in all individuals, but higher dose of aspirin can achieve complete inhibition of platelet aggregation (Helgason, Bolin et al. 1994). Higher dose of aspirin (325mg/day) were found better than lower dose (ten Berg, Gerritsen et al. 2002; Lim, Ali et al. 2003) and could overcome “aspirin resistance” both in patients and healthy volunteers, although deleterious side effect may occur, such as gut bleeding (Serebruany, Steinhubl et al. 2005). Actually, patients with high aspirin hydrolytic activity may lead to “aspirin resistance”, while patients with low aspirin hydrolytic activity may be prone to have bleeding problem (Gorelick and Weisman 2005). “Aspirin resistance” may be more predominant because low dose aspirin is commonly used. Optimal dosage of aspirin should be personalized (He, Whelton et al. 1998; 2010).

The final aim in medicine will be Personalized Medicine. There are always different requirements or reactions of patients to certain medicine or therapies. However, in Evidence Based Medicine (EBM), the current format of medicine, the doses of drug needed to treat patients are based on average treatment effects, which are fixed as a golden standard. In the other word, we treat one patient by using experiences from other patients. This experiences or data from studying group of population are applied to statistical models, which are used to evaluate certain risks and to make certain baseline to determine the “personalized” probability of benefit. However, the overall average degree of benefit for a statistical models is often more than that in any typical patient needs to be treated (Kent and Hayward 2007). The proposed baseline risk, which determines the benefits of patients, is usually highly skewed. So only a few high-risk patients, but not most patients, may have the power to determine most of the benefit and baseline risks (Ioannidis and Lau 1997). This situation is remarkable, when the overall risk of the outcome of interest is low. To avoid this kind of disadvantage, we need to change population-based analysis to individualized decision-making personalized medicine, which can apply an individual's unique biological information to optimize individual medical care. It is straightforward, but its implementation remains challenging.

The rising of pharmacogenetics makes personalized medicine possible and more reality by improving all aspects of its process. Pharmacogenetics refers to science dealing with how genetic variation can alter an individual's response to a certain therapy and medication or modify pharmacological properties of a certain drug.

This new field is a bridge which connects genetics to pharmacology. If genetic information is applied to genotype-specific investigations or incorporated into clinical trials successfully, we may be able to identify patients who would obtain the greatest overall benefit from the medication or therapy and accelerate process of drug development. For example, advances in pharmacogenomics of vascular biology have been applied to medical therapy of an individual's vascular diseases successfully (Degoma, Rivera et al. 2011).

As a part of cardiovascular medicine, oral anti-platelet therapy needs to be personalized. To understand and to perform personalized patient care in cardiovascular diseases, pharmacogenetics becomes more important in anti-platelet therapy field. However, genetic testing used to predict patient's response to various anti-platelet therapies and to determine drug or dose selection is more far from routine clinical use, although widely acknowledgement of it. However, pharmacogenetic-based selection of anti-platelet therapy will be supported by ongoing and future clinical trials, which provide the valuable evidence and data.

Aspirin as an established anti-platelet therapy is the therapeutic milestone for the prophylaxes of cardiovascular diseases in patients (Smith, Allen et al. 2006; Bhatt 2007). Aspirin irreversibly inactivates the cyclooxygenase-1 (COX-1) in platelets which cannot convert arachidonic acid to prostaglandin G_2 (PGG₂), blocking thromboxane A₂ (TXA₂) production, and then inhibits platelet activation. It is widely believed benefits of aspirin for primary CVD prevention are dependent on a patient's

baseline risk of CVD. However, therapeutic efficacy of aspirin varies between 5% and 60% depending on the population and methodology used (Gum, Kottke-Marchant et al. 2001; Patrono 2003; Gorog, Sweeny et al. 2009), namely ‘aspirin resistance’ which is highly controversial. A common understanding of aspirin resistance is that normal therapeutic amount of aspirin cannot inhibit platelet activation (Faraday, Yanek et al. 2007). Tests that directly measure COX-1 inhibition demonstrated 81 mg aspirin per day is adequate. But because of insufficient aspirin dosing and non-compliance, the so-called “resistance” might also occur in COX-1-dependent anti-platelet function of aspirin (Faraday, Becker et al. 2007; Faraday, Yanek et al. 2007) (Gurbel, Bliden et al. 2007). However, platelet agonists such as collagen, adenosine diphosphate (ADP), epinephrine, and thrombin, which may act via COX-1 independent pathways also results numerous biological heritability and variability in aspirin treated platelets (Faraday, Yanek et al. 2007; Gurbel, Bliden et al. 2007). Also in aspirin-treated patients, arachidonic acid (AA) still can induce platelet activation, which suggested via COX-independent pathways of platelet aggregation (Frelinger, Furman et al. 2006). All these indicate that platelet activation is a complicated event. Patients receiving therapeutic doses of aspirin still can suffer in severe cardiovascular events, which raised the issue of aspirin resistance (Maree, Curtin et al. 2005; Wang, Bhatt et al. 2006). To reveal the mechanism behind this, Sussman *et al* concluded that a single threshold for aspirin is inappropriate (Pearson, Blair et al. 2002; 2009). So to decide the right doses of aspirin a patient should take requires a robust and realistic

discussion of correlation between patients and drugs, including not only pharmacology but also the individualized risk assessment for CVD of patients.

All my work has been here provides not only basic molecular mechanisms of aspirin resistance, but also valuable pharmacogenetics data of aspirin metabolism. This is the first step from traditional EVB of aspirin to its personalized therapeutics.

7. BIBLIOGRAPHY

- (2009). "Aspirin for the prevention of cardiovascular disease: U.S. Preventive Services Task Force recommendation statement." Ann Intern Med **150**(6): 396-404.
- (2010). "Aspirin and primary cardiovascular prevention. Uncertain balance between benefits and risks." Prescrire Int **19**(110): 258-261.
- Adebayo, G. I., J. Williams, et al. (2007). "Aspirin esterase activity - Evidence for skewed distribution in healthy volunteers." Eur J Intern Med **18**(4): 299-303.
- Akopov, S. S., G. S. Grigorian, et al. (1992). "Dose-dependent aspirin hydrolysis and platelet aggregation in patients with atherosclerosis." J Clin Pharmacol **32**(2): 133-135.
- Alberts, M. J., D. L. Bergman, et al. (2004). "Antiplatelet effect of aspirin in patients with cerebrovascular disease." Stroke **35**(1): 175-178.
- Aldridge, W. N. (1953). "Serum esterases. I. Two types of esterase (A and B) hydrolysing p-nitrophenyl acetate, propionate and butyrate, and a method for their determination." Biochem J **53**(1): 110-117.
- Ali, B. and S. Kaur (1983). "Mammalian tissue acetylsalicylic acid esterase(s): identification, distribution and discrimination from other esterases." J Pharmacol Exp Ther **226**(2): 589-594.
- Arai, H., H. Koizumi, et al. (2002). "Platelet-activating factor acetylhydrolase (PAF-AH)." J Biochem **131**(5): 635-640.
- Augustinsson, K.-B. (1961). "Multiple Forms of Esterase in Vertebrate Blood Plasma." Annals of the New York Academy of Sciences **94**(3): 844-860.
- Awtry, E. H. and J. Loscalzo (2000). "Aspirin." Circulation **101**(10): 1206-1218.
- Baigent, C., L. Blackwell, et al. (2009). "Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials." Lancet **373**(9678): 1849-1860.
- Benveniste, J., M. Tence, et al. (1979). "[Semi-synthesis and proposed structure of platelet-activating factor (P.A.F.): PAF-acether an alkyl ether analog of lysophosphatidylcholine]." C R Seances Acad Sci D **289**(14): 1037-1040.
- Berglund, U. and L. Wallentin (1991). "Persistent inhibition of platelet function during long-term treatment with 75 mg acetylsalicylic acid daily in men with unstable coronary artery disease." Eur Heart J **12**(3): 428-433.
- Bergmann, F. and M. Wurzel (1954). "The structure of the active surface of serum cholinesterase." Biochim Biophys Acta **13**(2): 251-259.
- Bertele, V., M. Tomasiak, et al. (1982). "Aspirin inhibits platelet aggregation but not because it prevents thromboxane synthesis." Lancet **2**(8301): 775.
- Bhatt, D. L. (2007). "Intensifying platelet inhibition--navigating between Scylla and Charybdis." N Engl J Med **357**(20): 2078-2081.
- Bigler, J., J. Whitton, et al. (2001). "CYP2C9 and UGT1A6 genotypes modulate the protective effect of aspirin on colon adenoma risk." Cancer Res **61**(9): 3566-3569.

- Blank, M. L., F. Snyder, et al. (1979). "Antihypertensive activity of an alkyl ether analog of phosphatidylcholine." Biochem Biophys Res Commun **90**(4): 1194-1200.
- Blatter-Garin, M. C., B. Kalix, et al. (2003). "Aspirin use is associated with higher serum concentrations of the anti-oxidant enzyme, paraoxonase-1." Diabetologia **46**(4): 593-594.
- Blencowe, C., A. Hermetter, et al. (1995). "Enhanced association of platelet-activating factor acetylhydrolase with lipoprotein (a) in comparison with low density lipoprotein." J Biol Chem **270**(52): 31151-31157.
- Bonin, F., S. D. Ryan, et al. (2004). "Anti-apoptotic actions of the platelet-activating factor acetylhydrolase I alpha2 catalytic subunit." J Biol Chem **279**(50): 52425-52436.
- Bradbury, J. (2002). "Aspirin resistance may increase death risk in some patients with heart disease." Lancet **359**(9312): 1128.
- Braquet, P., R. Bourgain, et al. (1989). "Effect of platelet-activating factor on platelets and vascular endothelium." Semin Thromb Hemost **15**(2): 184-196.
- Braquet, P., L. Touqui, et al. (1987). "Perspectives in platelet-activating factor research." Pharmacol Rev **39**(2): 97-145.
- Brezinski, D. A., R. W. Nesto, et al. (1992). "Angioplasty triggers intracoronary leukotrienes and lipoxin A4. Impact of aspirin therapy." Circulation **86**(1): 56-63.
- Bridges, K. R., G. J. Schmidt, et al. (1975). "The acetylation of hemoglobin by aspirin. In vitro and in vivo." J Clin Invest **56**(1): 201-207.
- Buchanan, M. R. and S. J. Brister (1995). "Individual variation in the effects of ASA on platelet function: implications for the use of ASA clinically." Can J Cardiol **11**(3): 221-227.
- Burch, J. W. and B. Blazer-Yost (1981). "Acetylation of albumin by low doses of aspirin." Thromb Res **23**(4-5): 447-452.
- Byrn, S. R. and P. Y. Siew (1981). "Crystal structure and solid-state behavior of aspirin anhydride crystals." J Pharm Sci **70**(3): 280-283.
- Cattaneo, M. (2004). "Aspirin and clopidogrel: efficacy, safety, and the issue of drug resistance." Arterioscler Thromb Vasc Biol **24**(11): 1980-1987.
- Chan, A. T., G. J. Tranah, et al. (2005). "Genetic variants in the UGT1A6 enzyme, aspirin use, and the risk of colorectal adenoma." J Natl Cancer Inst **97**(6): 457-460.
- Chao, W. and M. S. Olson (1993). "Platelet-activating factor: receptors and signal transduction." Biochem J **292** (Pt 3): 617-629.
- Chapuis, N., C. Bruhlmann, et al. (2001). "The esterase-like activity of serum albumin may be due to cholinesterase contamination." Pharm Res **18**(10): 1435-1439.
- Chen, J., L. Yang, et al. (2007). "Intracellular PAF catabolism by PAF acetylhydrolase counteracts continual PAF synthesis." J Lipid Res **48**(11): 2365-2376.

- Costello, P. B., J. A. Caruana, et al. (1984). "The relative roles of hydrolases of the erythrocyte and other tissues in controlling aspirin survival in vivo." Arthritis Rheum **27**(4): 422-426.
- Costello, P. B. and F. A. Green (1982). "Aspirin survival in human blood modulated by the concentration of erythrocytes." Arthritis Rheum **25**(5): 550-555.
- Costello, P. B. and F. A. Green (1983). "Identification and partial purification of the major aspirin hydrolyzing enzyme in human blood." Arthritis Rheum **26**(4): 541-547.
- Costello, P. B. and F. A. Green (1986). "Patterns of in vitro aspirin hydrolysis rates in rheumatoid arthritis and systemic lupus erythematosus." J Rheumatol **13**(5): 882-886.
- Costello, P. B. and F. A. Green (1987). "The extracellular control of intracellular aspirin hydrolysis." Arthritis Rheum **30**(4): 412-418.
- Cox, D., A. O. Maree, et al. (2006). "Effect of enteric coating on antiplatelet activity of low-dose aspirin in healthy volunteers." Stroke **37**(8): 2153-2158.
- de Gaetano, G. (2004). "Aspirin resistance in diabetic patients." Diabetes Care **27**(5): 1244-1245; author reply 1246.
- Degoma, E. M., G. Rivera, et al. (2011). "Personalized vascular medicine: individualizing drug therapy." Vasc Med **16**(5): 391-404.
- Demopoulos, C. A., R. N. Pinckard, et al. (1979). "Platelet-activating factor. Evidence for 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine as the active component (a new class of lipid chemical mediators)." J Biol Chem **254**(19): 9355-9358.
- Dennis, E. A. (1997). "The growing phospholipase A2 superfamily of signal transduction enzymes." Trends Biochem Sci **22**(1): 1-2.
- Done, A. K. (1960). "Salicylate intoxication. Significance of measurements of salicylate in blood in cases of acute ingestion." Pediatrics **26**: 800-807.
- Elwood, P. C., A. L. Cochrane, et al. (1974). "A randomized controlled trial of acetyl salicylic acid in the secondary prevention of mortality from myocardial infarction." Br Med J **1**(5905): 436-440.
- Faraday, N., D. M. Becker, et al. (2007). "Pharmacogenomics of platelet responsiveness to aspirin." Pharmacogenomics **8**(10): 1413-1425.
- Faraday, N., L. R. Yanek, et al. (2007). "Heritability of platelet responsiveness to aspirin in activation pathways directly and indirectly related to cyclooxygenase-1." Circulation **115**(19): 2490-2496.
- Fiorucci, S., O. M. de Lima, Jr., et al. (2002). "Cyclooxygenase-2-derived lipoxin A4 increases gastric resistance to aspirin-induced damage." Gastroenterology **123**(5): 1598-1606.
- Fitzgerald, D. J. and A. Maree (2007). "Aspirin and clopidogrel resistance." Hematology Am Soc Hematol Educ Program: 114-120.
- Fitzgerald, D. J., L. Roy, et al. (1986). "Platelet activation in unstable coronary disease." N Engl J Med **315**(16): 983-989.
- FitzGerald, G. A. (1991). "Mechanisms of platelet activation: thromboxane A2 as an amplifying signal for other agonists." Am J Cardiol **68**(7): 11B-15B.

- FitzGerald, G. A. (2003). "Parsing an enigma: the pharmacodynamics of aspirin resistance." Lancet **361**(9357): 542-544.
- FitzGerald, G. A., R. L. Maas, et al. (1983). "Aspirin inhibits endogenous prostacyclin and thromboxane biosynthesis in man." Adv Prostaglandin Thromboxane Leukot Res **11**: 265-266.
- Fitzgerald, L. A., M. Poncz, et al. (1987). "Comparison of cDNA-derived protein sequences of the human fibronectin and vitronectin receptor alpha-subunits and platelet glycoprotein IIb." Biochemistry **26**(25): 8158-8165.
- Fontana, P., S. Nolli, et al. (2006). "Biological effects of aspirin and clopidogrel in a randomized cross-over study in 96 healthy volunteers." J Thromb Haemost **4**(4): 813-819.
- Frelinger, A. L., 3rd, M. I. Furman, et al. (2006). "Residual arachidonic acid-induced platelet activation via an adenosine diphosphate-dependent but cyclooxygenase-1- and cyclooxygenase-2-independent pathway: a 700-patient study of aspirin resistance." Circulation **113**(25): 2888-2896.
- Gandhi, P. N., X. Wang, et al. (2008). "The Roc domain of leucine-rich repeat kinase 2 is sufficient for interaction with microtubules." J Neurosci Res **86**(8): 1711-1720.
- Gawaz, M. (2004). "Role of platelets in coronary thrombosis and reperfusion of ischemic myocardium." Cardiovasc Res **61**(3): 498-511.
- Goodall, R. (2004). "Cholinesterase: phenotyping and genotyping." Ann Clin Biochem **41**(Pt 2): 98-110.
- Gorelick, P. B. and S. M. Weisman (2005). "Risk of hemorrhagic stroke with aspirin use: an update." Stroke **36**(8): 1801-1807.
- Gorog, D. A., J. M. Sweeny, et al. (2009). "Antiplatelet drug 'resistance'. Part 2: laboratory resistance to antiplatelet drugs-fact or artifact?" Nat Rev Cardiol **6**(5): 365-373.
- Greenberg, S., F. Di Virgilio, et al. (1988). "Extracellular nucleotides mediate Ca²⁺ fluxes in J774 macrophages by two distinct mechanisms." J Biol Chem **263**(21): 10337-10343.
- Gresner, P., M. Dolnik, et al. (2006). "Increased blood plasma hydrolysis of acetylsalicylic acid in type 2 diabetic patients: a role of plasma esterases." Biochim Biophys Acta **1760**(2): 207-215.
- Grotemeyer, K. H. (1991). "Effects of acetylsalicylic acid in stroke patients. Evidence of nonresponders in a subpopulation of treated patients." Thromb Res **63**(6): 587-593.
- Grotemeyer, K. H., H. W. Scharafinski, et al. (1993). "Two-year follow-up of aspirin responder and aspirin non responder. A pilot-study including 180 post-stroke patients." Thromb Res **71**(5): 397-403.
- Gum, P. A., K. Kottke-Marchant, et al. (2001). "Profile and prevalence of aspirin resistance in patients with cardiovascular disease." Am J Cardiol **88**(3): 230-235.

- Gurbel, P. A., K. P. Bliden, et al. (2007). "Evaluation of dose-related effects of aspirin on platelet function: results from the Aspirin-Induced Platelet Effect (ASPECT) study." Circulation **115**(25): 3156-3164.
- Harthorn, L. and M. Hedstrom (1971). "Hydrolysis of salicylsalicylic acid in human blood and plasma: a comparison with acetylsalicylic acid." Acta Pharmacol Toxicol (Copenh) **29**(23): 155-163.
- Hartwig-Otto, H. (1983). "Pharmacokinetic considerations of common analgesics and antipyretics." Am J Med **75**(5A): 30-37.
- Hattori, K., M. Hattori, et al. (1995). "Purification and characterization of platelet-activating factor acetylhydrolase II from bovine liver cytosol." J Biol Chem **270**(38): 22308-22313.
- Hattori, M., H. Adachi, et al. (1994). "The catalytic subunit of bovine brain platelet-activating factor acetylhydrolase is a novel type of serine esterase." J Biol Chem **269**(37): 23150-23155.
- Hattori, M., H. Adachi, et al. (1994). "The catalytic subunit of bovine brain platelet-activating factor acetylhydrolase is a novel type of serine esterase." Journal of Biological Chemistry **269**: 23150-23155.
- Hattori, M., H. Adachi, et al. (1994). "Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor." Nature **370**: 216-218.
- Hattori, M., H. Arai, et al. (1993). "Purification and characterization of bovine brain platelet-activating factor acetylhydrolase." Journal of Biological Chemistry **268**: 18748-18753.
- Hawkey, C. J. (2005). "COX-2 chronology." Gut **54**(11): 1509-1514.
- He, J., P. K. Whelton, et al. (1998). "Aspirin and risk of hemorrhagic stroke: a meta-analysis of randomized controlled trials." JAMA **280**(22): 1930-1935.
- Helgason, C. M., K. M. Bolin, et al. (1994). "Development of aspirin resistance in persons with previous ischemic stroke." Stroke **25**(12): 2331-2336.
- Helgason, C. M., J. A. Hoff, et al. (1993). "Platelet aggregation in patients with atrial fibrillation taking aspirin or warfarin." Stroke **24**(10): 1458-1461.
- Helgason, C. M., K. L. Tortorice, et al. (1993). "Aspirin response and failure in cerebral infarction." Stroke **24**(3): 345-350.
- Hennekens, C. H., K. Schror, et al. (2004). "Terms and conditions: semantic complexity and aspirin resistance." Circulation **110**(12): 1706-1708.
- Higgs, G. A., J. A. Salmon, et al. (1987). "Pharmacokinetics of aspirin and salicylate in relation to inhibition of arachidonate cyclooxygenase and antiinflammatory activity." Proc Natl Acad Sci U S A **84**(5): 1417-1420.
- Ho, Y. S., P. J. Sheffield, et al. (1999). "Probing the substrate specificity of the intracellular brain platelet-activating factor acetylhydrolase." Protein Eng **12**(8): 693-700.
- Ho, Y. S., L. Swenson, et al. (1997). "Brain acetylhydrolase that inactivates platelet-activating factor is a G-protein-like trimer." Nature **385**(6611): 89-93.
- Hollister, L. and G. Levy (1965). "Some aspects of salicylate distribution and metabolism in man." J Pharm Sci **54**(8): 1126-1129.

- Honda, Z., M. Nakamura, et al. (1991). "Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung." Nature **349**(6307): 342-346.
- Horsch, W. (1979). "[The salicylates]." Pharmazie **34**(9): 585-604.
- Houslay, M. D., D. Bojanic, et al. (1986). "Platelet activating factor and U44069 stimulate a GTPase activity in human platelets which is distinct from the guanine nucleotide regulatory proteins, Ns and Ni." Biochem J **234**(3): 737-740.
- Hwang, S. B., M. H. Lam, et al. (1986). "Ionic and GTP regulation of binding of platelet-activating factor to receptors and platelet-activating factor-induced activation of GTPase in rabbit platelet membranes." J Biol Chem **261**(2): 532-537.
- Ioannidis, J. P. and J. Lau (1997). "The impact of high-risk patients on the results of clinical trials." J Clin Epidemiol **50**(10): 1089-1098.
- Ishii, S. and T. Shimizu (2000). "Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice." Prog Lipid Res **39**(1): 41-82.
- Jack, D. B. (1997). "One hundred years of aspirin." Lancet **350**(9075): 437-439.
- Jaichander, P., K. Selvarajan, et al. (2008). "Induction of paraoxonase 1 and apolipoprotein A-I gene expression by aspirin." J Lipid Res **49**(10): 2142-2148.
- Karasawa, K., M. Shirakura, et al. (2005). "Red blood cells highly express type I platelet-activating factor-acetylhydrolase (PAF-AH) which consists of the alpha1/alpha2 complex." J Biochem **138**(4): 509-517.
- Kent, D. M. and R. A. Hayward (2007). "Limitations of applying summary results of clinical trials to individual patients: the need for risk stratification." JAMA **298**(10): 1209-1212.
- Kim, Y. and K. Machida (1986). "Vibrational spectra, normal coordinates and infrared intensities of aspirin crystal." Chem Pharm Bull (Tokyo) **34**(8): 3087-3096.
- Klopprogge, E. and J. W. Akkerman (1984). "Binding kinetics of PAF-acether (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) to intact human platelets." Biochem J **223**(3): 901-909.
- Koizumi, H., N. Yamaguchi, et al. (2003). "Targeted disruption of intracellular type I platelet activating factor-acetylhydrolase catalytic subunits causes severe impairment in spermatogenesis." J Biol Chem **278**(14): 12489-12494.
- Kotani, K., S. Kimura, et al. (2010). "Serum aspirin esterase is strongly associated with glucose and lipids in healthy subjects: different association patterns in subjects with type 2 diabetes mellitus." Diabetol Metab Syndr **2**: 50.
- Kriska, T., G. K. Marathe, et al. (2007). "Phospholipase action of platelet-activating factor acetylhydrolase, but not paraoxonase-1, on long fatty acyl chain phospholipid hydroperoxides." J Biol Chem **282**(1): 100-108.
- Kurono, Y., T. Kondo, et al. (1983). "Esterase-like activity of human serum albumin: enantioselectivity in the burst phase of reaction with p-nitrophenyl alpha-methoxyphenyl acetate." Arch Biochem Biophys **227**(1): 339-341.

- La Du, B. (1971). "Plasma esterase activity and the metabolism of drugs with ester groups." Ann N Y Acad Sci **179**: 684-694.
- Lapetina, E. G. and F. L. Siegel (1983). "Shape change induced in human platelets by platelet-activating factor. Correlation with the formation of phosphatidic acid and phosphorylation of a 40,000-dalton protein." J Biol Chem **258**(12): 7241-7244.
- Lepantalo, A., J. Mikkelsson, et al. (2006). "Polymorphisms of COX-1 and GPVI associate with the antiplatelet effect of aspirin in coronary artery disease patients." Thromb Haemost **95**(2): 253-259.
- Lev, E. I. (2009). "Aspirin resistance transient laboratory finding or important clinical entity?" J Am Coll Cardiol **53**(8): 678-680.
- Levano, S., H. Ginz, et al. (2005). "Genotyping the butyrylcholinesterase in patients with prolonged neuromuscular block after succinylcholine." Anesthesiology **102**(3): 531-535.
- Levy, G. (1965). "Aspirin, absorption rate and analgesic effect." Anesth Analg **44**(6): 837-841.
- Levy, G. (1965). "Pharmacokinetics of salicylate elimination in man." J Pharm Sci **54**(7): 959-967.
- Levy, G. (1978). "Clinical pharmacokinetics of aspirin." Pediatrics **62**(5 Pt 2 Suppl): 867-872.
- Levy, G. (1979). "Pharmacokinetics of salicylate in man." Drug Metab Rev **9**(1): 3-19.
- Levy, G. and T. Tsuchiya (1972). "Effect of activated charcoal on aspirin absorption in man. Part I." Clin Pharmacol Ther **13**(3): 317-322.
- Li, B., M. Sedlacek, et al. (2005). "Butyrylcholinesterase, paraoxonase, and albumin esterase, but not carboxylesterase, are present in human plasma." Biochem Pharmacol **70**(11): 1673-1684.
- Liddell, J., H. Lehmann, et al. (1962). "A 'silent' pseudo-cholinesterase gene." Nature **193**: 561-562.
- Lim, E., Z. Ali, et al. (2003). "Indirect comparison meta-analysis of aspirin therapy after coronary surgery." BMJ **327**(7427): 1309.
- Liyasova, M. S., L. M. Schopfer, et al. (2009). "Reaction of human albumin with aspirin in vitro: mass spectrometric identification of acetylated lysines 199, 402, 519, and 545." Biochem Pharmacol.
- Liyasova, M. S., L. M. Schopfer, et al. (2010). "Reaction of human albumin with aspirin in vitro: mass spectrometric identification of acetylated lysines 199, 402, 519, and 545." Biochem Pharmacol **79**(5): 784-791.
- Lockridge, O., S. Adkins, et al. (1987). "Location of disulfide bonds within the sequence of human serum cholinesterase." J Biol Chem **262**(27): 12945-12952.
- Lockridge, O., W. Xue, et al. (2008). "Pseudo-esterase activity of human albumin: slow turnover on tyrosine 411 and stable acetylation of 82 residues including 59 lysines." J Biol Chem **283**(33): 22582-22590.
- Macchi, L., L. Christiaens, et al. (2002). "Resistance to aspirin in vitro is associated with increased platelet sensitivity to adenosine diphosphate." Thromb Res **107**(1-2): 45-49.

- MacIntyre, D. E. and W. K. Pollock (1983). "Platelet-activating factor stimulates phosphatidylinositol turnover in human platelets." Biochem J **212**(2): 433-437.
- Mackness, M. I., S. D. Hallam, et al. (1985). "The separation of sheep and human serum "A"-esterase activity into the lipoprotein fraction by ultracentrifugation." Comp Biochem Physiol B **82**(4): 675-677.
- Maclagan, T. (1876). "The treatment of acute rheumatism by salicin." Lancet **2**: 4.
- Maclouf, J., G. Folco, et al. (1998). "Eicosanoids and iso-eicosanoids: constitutive, inducible and transcellular biosynthesis in vascular disease." Thromb Haemost **79**(4): 691-705.
- Manya, H., J. Aoki, et al. (1999). "Biochemical characterization of various catalytic complexes of the brain platelet-activating factor acetylhydrolase." J Biol Chem **274**(45): 31827-31832.
- Manya, H., J. Aoki, et al. (1998). "Switching of platelet-activating factor acetylhydrolase catalytic subunits in developing rat brain." J Biol Chem **273**(29): 18567-18572.
- Marathe, G. K., G. A. Zimmerman, et al. (2003). "Platelet-activating factor acetylhydrolase, and not paraoxonase-1, is the oxidized phospholipid hydrolase of high density lipoprotein particles." J Biol Chem **278**(6): 3937-3947.
- Maree, A. O., D. Cox, et al. (2007). "Drug insight: aspirin resistance-fact or fashion?" Nat Clin Pract Cardiovasc Med **4**(3): E1; author reply E2.
- Maree, A. O., R. J. Curtin, et al. (2005). "Cyclooxygenase-1 haplotype modulates platelet response to aspirin." J Thromb Haemost **3**(10): 2340-2345.
- Marshall, P. W., A. J. Williams, et al. (1997). "A comparison of the effects of aspirin on bleeding time measured using the Simplate method and closure time measured using the PFA-100, in healthy volunteers." Br J Clin Pharmacol **44**(2): 151-155.
- Mason, P. J., A. K. Jacobs, et al. (2005). "Aspirin resistance and atherothrombotic disease." J Am Coll Cardiol **46**(6): 986-993.
- Masson, P., M. T. Froment, et al. (1998). "Butyrylcholinesterase-catalysed hydrolysis of aspirin, a negatively charged ester, and aspirin-related neutral esters." Biochim Biophys Acta **1387**(1-2): 41-52.
- McIntyre, T. M., S. M. Prescott, et al. (2009). "The emerging roles of PAF acetylhydrolase." J Lipid Res **50** Suppl: S255-259.
- McKee, S. A., D. C. Sane, et al. (2002). "Aspirin resistance in cardiovascular disease: a review of prevalence, mechanisms, and clinical significance." Thromb Haemost **88**(5): 711-715.
- Mentlein, R. and E. Heymann (1984). "Hydrolysis of ester- and amide-type drugs by the purified isoenzymes of nonspecific carboxylesterase from rat liver." Biochem Pharmacol **33**(8): 1243-1248.
- Miner, J. and A. Hoffhines (2007). "The discovery of aspirin's antithrombotic effects." Tex Heart Inst J **34**(2): 179-186.

- Mochizuki, H., S. W. Scherer, et al. (1998). "Human PON2 gene at 7q21.3: cloning, multiple mRNA forms, and missense polymorphisms in the coding sequence." Gene **213**(1-2): 149-157.
- Morikawa, M., M. Inoue, et al. (1979). "Studies on aspirin esterase of human serum." Jpn J Pharmacol **29**(4): 581-586.
- Morton, R. E. and T. A. Evans (1992). "Modification of the bicinchoninic acid protein assay to eliminate lipid interference in determining lipoprotein protein content." Anal Biochem **204**(2): 332-334.
- Mueller, M. R., A. Salat, et al. (1997). "Variable platelet response to low-dose ASA and the risk of limb deterioration in patients submitted to peripheral arterial angioplasty." Thromb Haemost **78**(3): 1003-1007.
- Needs, C. J. and P. M. Brooks (1985). "Clinical pharmacokinetics of the salicylates." Clin Pharmacokinet **10**(2): 164-177.
- Ninio, E., S. Leyravaud, et al. (1991). "Cell adhesion by membrane-bound paf-acether." Int Immunol **3**(11): 1157-1163.
- Nogueira, C. P., M. C. McGuire, et al. (1990). "Identification of a frameshift mutation responsible for the silent phenotype of human serum cholinesterase, Gly 117 (GGT---GGAG)." Am J Hum Genet **46**(5): 934-942.
- Nothwang, H. G., H. G. Kim, et al. (2001). "Functional hemizygoty of PAFAH1B3 due to a PAFAH1B3-CLK2 fusion gene in a female with mental retardation, ataxia and atrophy of the brain." Hum Mol Genet **10**(8): 797-806.
- O'Brien, J. R. (1968). "Effects of salicylates on human platelets." Lancet **1**(7546): 779-783.
- Ohsako, M., Y. Matsumoto, et al. (1993). "Transport of aspirin and its metabolites through human erythrocyte membrane." Biol Pharm Bull **16**(2): 154-157.
- Ostrovsky, L., A. J. King, et al. (1998). "A juxtacrine mechanism for neutrophil adhesion on platelets involves platelet-activating factor and a selectin-dependent activation process." Blood **91**(8): 3028-3036.
- Page, C. and A. Abbott (1989). "PAF: new antagonists, new roles in disease and a major role in reproductive biology." Trends Pharmacol Sci **10**(7): 255-257.
- Pappas, J. M., J. C. Westengard, et al. (1994). "Population variability in the effect of aspirin on platelet function. Implications for clinical trials and therapy." Arch Pathol Lab Med **118**(8): 801-804.
- Patrono, C. (2003). "Aspirin resistance: definition, mechanisms and clinical read-outs." J Thromb Haemost **1**(8): 1710-1713.
- Patrono, C. and B. Rocca (2007). "Drug insight: aspirin resistance--fact or fashion?" Nat Clin Pract Cardiovasc Med **4**(1): 42-50.
- Pearson, T. A., S. N. Blair, et al. (2002). "AHA Guidelines for Primary Prevention of Cardiovascular Disease and Stroke: 2002 Update: Consensus Panel Guide to Comprehensive Risk Reduction for Adult Patients Without Coronary or Other Atherosclerotic Vascular Diseases. American Heart Association Science Advisory and Coordinating Committee." Circulation **106**(3): 388-391.

- Pedersen, A. K. and G. A. FitzGerald (1984). "Dose-related kinetics of aspirin. Presystemic acetylation of platelet cyclooxygenase." N Engl J Med **311**(19): 1206-1211.
- Pierige, F., S. Serafini, et al. (2008). "Cell-based drug delivery." Adv Drug Deliv Rev **60**(2): 286-295.
- Pignone, M. and C. D. Williams (2010). "Aspirin for primary prevention of cardiovascular disease in diabetes mellitus." Nat Rev Endocrinol **6**(11): 619-628.
- Pinckard, R. N., D. Hawkins, et al. (1968). "In vitro acetylation of plasma proteins, enzymes and DNA by aspirin." Nature **219**(5149): 68-69.
- Primo-Parmo, S. L., R. C. Sorenson, et al. (1996). "The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family." Genomics **33**(3): 498-507.
- Puche, E., E. Gomez-Valverde, et al. (1993). "Postoperative decline in plasma aspirin-esterase and cholinesterase activity in surgical patients." Acta Anaesthesiol Scand **37**(1): 20-22.
- Pulcinelli, F. M., P. Pignatelli, et al. (2004). "Inhibition of platelet aggregation by aspirin progressively decreases in long-term treated patients." J Am Coll Cardiol **43**(6): 979-984.
- Rainsford, K. D., N. L. Ford, et al. (1980). "Plasma aspirin esterases in normal individuals, patients with alcoholic liver disease and rheumatoid arthritis: characterization and the importance of the enzymic components." Eur J Clin Invest **10**(5): 413-420.
- Reddy, S. T., D. J. Wadleigh, et al. (2001). "Human paraoxonase-3 is an HDL-associated enzyme with biological activity similar to paraoxonase-1 protein but is not regulated by oxidized lipids." Arterioscler Thromb Vasc Biol **21**(4): 542-547.
- Redinbaugh, M. G. and W. H. Campbell (1985). "Adaptation of the dye-binding protein assay to microtiter plates." Anal Biochem **147**(1): 144-147.
- Redinbaugh, M. G. and R. B. Turley (1986). "Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions." Anal Biochem **153**(2): 267-271.
- Reilly, I. A. and G. A. FitzGerald (1988). "Aspirin in cardiovascular disease." Drugs **35**(2): 154-176.
- Ronald Hoffman; Edward J. Benz, J. S. J. S. B. F. (1991). Hematology: basic principles and practice, Churchill Livingstone Inc.
- Roth, G. J. and D. C. Calverley (1994). "Aspirin, platelets, and thrombosis: theory and practice." Blood **83**(4): 885-898.
- Roth, G. J. and P. W. Majerus (1975). "The mechanism of the effect of aspirin on human platelets. I. Acetylation of a particulate fraction protein." J Clin Invest **56**(3): 624-632.
- Roth, G. J., N. Stanford, et al. (1975). "Acetylation of prostaglandin synthase by aspirin." Proc Natl Acad Sci U S A **72**(8): 3073-3076.

- Rowland, M., S. Riegelman, et al. (1972). "Absorption kinetics of aspirin in man following oral administration of an aqueous solution." J Pharm Sci **61**(3): 379-385.
- Rylance, H. J. and R. C. Wallace (1981). "Erythrocyte and plasma aspirin esterase." Br J Clin Pharmacol **12**(3): 436-438.
- Sakurai, Y., S. F. Ma, et al. (2004). "Esterase-like activity of serum albumin: characterization of its structural chemistry using p-nitrophenyl esters as substrates." Pharm Res **21**(2): 285-292.
- Sans, S., H. Kesteloot, et al. (1997). "The burden of cardiovascular diseases mortality in Europe. Task Force of the European Society of Cardiology on Cardiovascular Mortality and Morbidity Statistics in Europe." Eur Heart J **18**(12): 1231-1248.
- Santanam, N. and S. Parthasarathy (2007). "Aspirin is a substrate for paraoxonase-like activity: implications in atherosclerosis." Atherosclerosis **191**(2): 272-275.
- Santilli, F., B. Rocca, et al. (2009). "Platelet cyclooxygenase inhibition by low-dose aspirin is not reflected consistently by platelet function assays: implications for aspirin "resistance"." J Am Coll Cardiol **53**(8): 667-677.
- Santos, M. T., A. Moscardo, et al. (2000). "Participation of tyrosine phosphorylation in cytoskeletal reorganization, alpha(IIb)beta(3) integrin receptor activation, and aspirin-insensitive mechanisms of thrombin-stimulated human platelets." Circulation **102**(16): 1924-1930.
- Santos, M. T., J. Valles, et al. (1997). "Prothrombotic effects of erythrocytes on platelet reactivity. Reduction by aspirin." Circulation **95**(1): 63-68.
- Saxena, A., W. Sun, et al. (2011). "Prophylaxis with human serum butyrylcholinesterase protects guinea pigs exposed to multiple lethal doses of soman or VX." Biochem Pharmacol **81**(1): 164-169.
- Schorr, K. (2002). "The pharmacology of cilostazol." Diabetes Obes Metab **4 Suppl 2**: S14-19.
- Schrör, K. (2009). Acetylsalicylic Acid, Wiley-VCH Verlag GmbH & Co. KGaA.
- Schorr, K., A. A. Weber, et al. (2006). "Aspirin "resistance"." Blood Cells Mol Dis **36**(2): 171-176.
- Schwartz, K. A., D. E. Schwartz, et al. (2005). "Compliance as a critical consideration in patients who appear to be resistant to aspirin after healing of myocardial infarction." Am J Cardiol **95**(8): 973-975.
- Serebruany, V. L., S. R. Steinhubl, et al. (2005). "Analysis of risk of bleeding complications after different doses of aspirin in 192,036 patients enrolled in 31 randomized controlled trials." Am J Cardiol **95**(10): 1218-1222.
- Seymour, R. A., F. M. Williams, et al. (1984). "Aspirin metabolism and efficacy in postoperative dental pain." Br J Clin Pharmacol **17**(6): 697-701.
- Shen, J., S. Wanwimolruk, et al. (1991). "Model representation of salicylate pharmacokinetics using unbound plasma salicylate concentrations and metabolite urinary excretion rates following a single oral dose." J Pharmacokinet Biopharm **19**(5): 575-595.

- Simeon-Rudolf, V., E. Reiner, et al. (1999). "Catalytic parameters for the hydrolysis of butyrylthiocholine by human serum butyrylcholinesterase variants." Chem Biol Interact **119-120**: 165-171.
- Six, D. A. and E. A. Dennis (2000). "The expanding superfamily of phospholipase A(2) enzymes: classification and characterization." Biochim Biophys Acta **1488**(1-2): 1-19.
- Smith, S. C., Jr., J. Allen, et al. (2006). "AHA/ACC guidelines for secondary prevention for patients with coronary and other atherosclerotic vascular disease: 2006 update: endorsed by the National Heart, Lung, and Blood Institute." Circulation **113**(19): 2363-2372.
- Smith, W. L., D. L. DeWitt, et al. (2000). "CYCLOOXYGENASES: structural, cellular, and molecular biology." Annu Rev Biochem **69**: 145-182.
- Smith, W. L., D. L. DeWitt, et al. (1990). "Structure-function relationships in sheep, mouse, and human prostaglandin endoperoxide G/H synthases." Adv Prostaglandin Thromboxane Leukot Res **20**: 14-21.
- Snyder, F. (1995). "Platelet-activating factor and its analogs: metabolic pathways and related intracellular processes." Biochim Biophys Acta **1254**(3): 231-249.
- Spenney, J. G. (1978). "Acetylsalicylic acid hydrolase of gastric mucosa." Am J Physiol **234**(6): E606-610.
- Stafforini, D. M., M. E. Carter, et al. (1989). "Lipoproteins alter the catalytic behavior of the platelet-activating factor acetylhydrolase in human plasma." Proc Natl Acad Sci U S A **86**(7): 2393-2397.
- Stafforini, D. M., M. R. Elstad, et al. (1990). "Human macrophages secrete platelet-activating factor acetylhydrolase." J Biol Chem **265**(17): 9682-9687.
- Stafforini, D. M., T. M. McIntyre, et al. (1987). "Human plasma platelet-activating factor acetylhydrolase. Association with lipoprotein particles and role in the degradation of platelet-activating factor." J Biol Chem **262**(9): 4215-4222.
- Stafforini, D. M., S. M. Prescott, et al. (1996). "Mammalian platelet-activating factor acetylhydrolases." Biochim Biophys Acta **1301**(3): 161-173.
- Stafforini, D. M., E. N. Rollins, et al. (1993). "The platelet-activating factor acetylhydrolase from human erythrocytes. Purification and properties." J Biol Chem **268**(6): 3857-3865.
- Stejskal, D., J. Proskova, et al. (2004). "[Use of assessment of aggregation of thrombocytes induced by cationic propyl gallate to estimate recurrence of cardiovascular complications]." Vnitr Lek **50**(8): 591-599.
- Stremmler, K. E., D. M. Stafforini, et al. (1991). "Human plasma platelet-activating factor acetylhydrolase. Oxidatively fragmented phospholipids as substrates." J Biol Chem **266**(17): 11095-11103.
- Stremmler, K. E., D. M. Stafforini, et al. (1989). "An oxidized derivative of phosphatidylcholine is a substrate for the platelet-activating factor acetylhydrolase from human plasma." J Biol Chem **264**(10): 5331-5334.
- Sung, S. S., J. D. Young, et al. (1985). "Extracellular ATP perturbs transmembrane ion fluxes, elevates cytosolic [Ca²⁺], and inhibits phagocytosis in mouse macrophages." J Biol Chem **260**(25): 13442-13449.

- Szczeklik, A., R. J. Gryglewski, et al. (1979). "Platelet aggregability, thromboxane A₂ and malonaldehyde formation following administration of aspirin to man." Thromb Res **15**(3-4): 405-413.
- Szczeklik, A., J. Musial, et al. (2002). "Reasons for resistance to aspirin in cardiovascular disease." Circulation **106**(22): e181-182; author reply e181-182.
- Sztriha, L. K., K. Sas, et al. (2005). "Aspirin resistance in stroke: 2004." J Neurol Sci **229-230**: 163-169.
- Takahashi, S., M. Ushida, et al. (2008). "Platelet responsiveness to in vitro aspirin is independent of COX-1 and COX-2 protein levels and polymorphisms." Thromb Res **121**(4): 509-517.
- Tantry, U. S., K. P. Bliden, et al. (2005). "Overestimation of platelet aspirin resistance detection by thrombelastograph platelet mapping and validation by conventional aggregometry using arachidonic acid stimulation." J Am Coll Cardiol **46**(9): 1705-1709.
- Tantry, U. S., E. Mahla, et al. (2009). "Aspirin resistance." Prog Cardiovasc Dis **52**(2): 141-152.
- ten Berg, J. M., W. B. Gerritsen, et al. (2002). "High-dose aspirin in addition to daily low-dose aspirin decreases platelet activation in patients before and after percutaneous coronary intervention." Thromb Res **105**(5): 385-390.
- Thun, M. J., M. M. Namboodiri, et al. (1991). "Aspirin use and reduced risk of fatal colon cancer." N Engl J Med **325**(23): 1593-1596.
- Tjoelker, L. W., C. Wilder, et al. (1995). "Anti-inflammatory properties of a platelet-activating factor acetylhydrolase." Nature **374**(6522): 549-553.
- Tohgi, H., S. Konno, et al. (1992). "Effects of low-to-high doses of aspirin on platelet aggregability and metabolites of thromboxane A₂ and prostacyclin." Stroke **23**(10): 1400-1403.
- Tselepis, A. D., C. Dentan, et al. (1995). "PAF-degrading acetylhydrolase is preferentially associated with dense LDL and VLDL-1 in human plasma. Catalytic characteristics and relation to the monocyte-derived enzyme." Arterioscler Thromb Vasc Biol **15**(10): 1764-1773.
- Ukena, D., G. Dent, et al. (1988). "Radioligand binding of antagonists of platelet-activating factor to intact human platelets." FEBS Lett **228**(2): 285-289.
- Undas, A., K. Brummel, et al. (2001). "PI(A₂) polymorphism of beta(3) integrins is associated with enhanced thrombin generation and impaired antithrombotic action of aspirin at the site of microvascular injury." Circulation **104**(22): 2666-2672.
- Valles, J., M. T. Santos, et al. (1998). "Erythrocyte promotion of platelet reactivity decreases the effectiveness of aspirin as an antithrombotic therapeutic modality: the effect of low-dose aspirin is less than optimal in patients with vascular disease due to prothrombotic effects of erythrocytes on platelet reactivity." Circulation **97**(4): 350-355.
- Valone, F. H. (1988). "Platelet-activating factor binding and metabolism during human platelet aggregation." Thromb Res **50**(1): 103-112.

- Vane, J. R. (1971). "Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs." Nat New Biol **231**(25): 232-235.
- Wang, J. C., D. Aucoin-Barry, et al. (2003). "Incidence of aspirin nonresponsiveness using the Ultegra Rapid Platelet Function Assay-ASA." Am J Cardiol **92**(12): 1492-1494.
- Wang, T. H., D. L. Bhatt, et al. (2006). "Aspirin and clopidogrel resistance: an emerging clinical entity." Eur Heart J **27**(6): 647-654.
- Warner, T. D. and J. A. Mitchell (2002). "Cyclooxygenase-3 (COX-3): filling in the gaps toward a COX continuum?" Proc Natl Acad Sci U S A **99**(21): 13371-13373.
- Warner, T. D., I. Vojnovic, et al. (2006). "Influence of plasma protein on the potencies of inhibitors of cyclooxygenase-1 and -2." FASEB J **20**(3): 542-544.
- Weber, A. A., B. Przytulski, et al. (2002). "Towards a definition of aspirin resistance: a typological approach." Platelets **13**(1): 37-40.
- Weber, A. A., K. C. Zimmermann, et al. (1999). "Cyclooxygenase-2 in human platelets as a possible factor in aspirin resistance." Lancet **353**(9156): 900.
- Weber, C. and T. A. Springer (1997). "Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to alphaIIb beta3 and stimulated by platelet-activating factor." J Clin Invest **100**(8): 2085-2093.
- Wei, Y., L. Swenson, et al. (1998). "Structure of a microbial homologue of mammalian platelet-activating factor acetylhydrolases: *Streptomyces exfoliatus* lipase at 1.9 Å resolution." Structure **6**(4): 511-519.
- Weiss, H. J. (2003). "The discovery of the antiplatelet effect of aspirin: a personal reminiscence." J Thromb Haemost **1**(9): 1869-1875.
- Weksler, B. B., J. L. Kent, et al. (1985). "Effects of low dose aspirin on platelet function in patients with recent cerebral ischemia." Stroke **16**(1): 5-9.
- Wessel, D. and U. I. Flugge (1984). "A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids." Anal Biochem **138**(1): 141-143.
- Weyrich, A. S., T. M. McIntyre, et al. (1995). "Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-alpha secretion. Signal integration and NF-kappa B translocation." J Clin Invest **95**(5): 2297-2303.
- Williams, F. M., S. I. Asad, et al. (1987). "Plasma esterase activity in patients with aspirin-sensitive asthma or urticaria." Eur J Clin Pharmacol **33**(4): 387-390.
- Williams, F. M., E. M. Mutch, et al. (1989). "Human liver and plasma aspirin esterase." J Pharm Pharmacol **41**(6): 407-409.
- Wiviott, S. D. and E. M. Antman (2004). "Clopidogrel resistance: a new chapter in a fast-moving story." Circulation **109**(25): 3064-3067.
- Yamaori, S., N. Fujiyama, et al. (2006). "Involvement of human blood arylesterases and liver microsomal carboxylesterases in nafamostat hydrolysis." Drug Metab Pharmacokinet **21**(2): 147-155.

- Yan, W., A. H. Assadi, et al. (2003). "Previously uncharacterized roles of platelet-activating factor acetylhydrolase 1b complex in mouse spermatogenesis." Proc Natl Acad Sci U S A **100**(12): 7189-7194.
- Yang, F., C. Bian, et al. (2007). "Effect of human serum albumin on drug metabolism: structural evidence of esterase activity of human serum albumin." J Struct Biol **157**(2): 348-355.
- Zandi, P. P. and J. C. Breitner (2001). "Do NSAIDs prevent Alzheimer's disease? And, if so, why? The epidemiological evidence." Neurobiol Aging **22**(6): 811-817.
- Zhou, W., M. A. Javors, et al. (1992). "Platelet-activating factor as an intercellular signal in neutrophil-dependent platelet activation." J Immunol **149**(5): 1763-1769.
- Zimrin, A. B., R. Eisman, et al. (1988). "Structure of platelet glycoprotein IIIa. A common subunit for two different membrane receptors." J Clin Invest **81**(5): 1470-1475.